SOME ABNORMALITIES OF PLATELET AND LEUCOCYTE FUNCTION, AND CHANGES IN FREE RADICAL ACTIVITY : THEIR POSSIBLE ROLES IN THE DEVELOPMENT OF DIABETIC MICROVASCULAR COMPLICATIONS

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DECLARATION

The studies in this thesis were designed and undertaken by myself and the composition is my own. Contributions and assistance from others are acknowledged. This thesis has not been submitted in canditature for any other degree, diploma or professional qualification.

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Interest in platelet research developed while an undergraduate working under the supervision of Professor E Horton in the Department of Pharmacology, University of Edinburgh. During that period I learned a number of techniques including platelet aggregation and radioimmunoassay. Later I had the opportunity of using these and other techniques for studying groups of diabetic patients while holding an appointment in the Diabetic Department in the Royal Infirmary, Edinburgh.

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ABSTRACT

Microangiopathy is a major cause of morbidity and mortality in diabetes mellitus, and its pathogenesis remains poorly understood. Abnormalities of both in vivo and in vitro haemostasis have been extensively reported, particularly changes in platelet function have been described in cross-sectional studies, and cause-end-effect relationships have been postulated. It is also suggested that neutrophil accumulation at sites of inflammation, including vascular endothelium, can induce tissue damage. Three classes of mediators are particularly likely to participate in the pathogenesis of neutrophil-mediated vascular injury : granule enzymes, reactive oxygen metabolites, and products of membrane phospholipids.

The aims of this thesis were to investigate changes in platelet function in insulin-dependent diabetic patients in relation to glycaemic control, and microvascular disease in the form of retinopathy. In addition, the changes in platelet function in non-insulin-dependent diabetic patients at diagnosis, and the effect of glycaemic control and oral hypoglycaemic therapy were assessed. As a measure of neutrophil activity, neutrophil elastase was measured in insulin-dependent diabetic patients with retinopathy. The changes in elastase levels in response to acute insulin-

induced hypoglycaemia in non-diabetic subjects were also evaluated. A further aim was to measure a marker for free radical activity in insulin-dependent diabetes.

The changes in platelet density, thromboxane production and receptor sensitivity demonstrated are consistent with platelets from diabetic patients being hyperaggregable and contributing towards microvascular disease. The increase in neutrophil elastase levels in insulin-dependent diabetic patients, and the rise in normals in response to hypoglycaemia are consistent with leucocyte-endothelial interaction contributing towards diabetic vascular disease. Although, the marker for free radical activity was not increased in the retinopathic insulin-dependent diabetic patients studied, it is probable that abnormalities of lipid metabolism related to free radical activity may still contribute to diabetic vascular disease.

Although good glycaemic control can delay the onset and slow the progress of diabetic complications, specific therapeutic agents that interfere with haemostasis, leucocyte function and free radical activity may also have a protective role in the development of diabetic complications. In addition, use of these agents may also give greater understanding of the aetiopathogenesis of diabetic microangiopathy.

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SECTION A

GENERAL BACKGROUND TO PLATELET, LEUCOCYTE, AND FREE RADICAL ACTIVITY IN NORMAL AND DIABETIC SUBJECTS

CHAPTER 1

GENERAL BACKGROUND AND AIMS

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1.1 Introduction

The introduction of insulin in 1922 was one of the great achievements of 20th century medicine; it provided a lifesaving remedy for many diabetic patients and led to a new era for diabetes. However, although many of the features of diabetic complications had been described well before the introduction of insulin it was not until patients survived for longer periods that their full extent and often fatal nature was realised. It was only in 1954 that Lundbaek introduced the concept of diabetic angiopathy (a specific widespread diabetic small vessel disease) as the common mechanism leading to diabetic complications.

Vascular disease remains the most common cause of morbidity in the diabetic population (Robertson and Strong, 1968). The pathogenesis of both macrovascular and microvascular disease in diabetes mellitus remains poorly understood. Macrovascular disease may present in the form of a thrombo-embolic event like myocardial infarction, peripheral vascular disease and cardiovascular disease. It often occurs earlier and advances more rapidly in diabetic patients than in the general population (Kannel and McGee, 1979; Ganda, 1980). Small vessel disease is unique to diabetes. Thickening of the basement membrane,

microthrombosis and endothelial proliferation are the main pathogenic features, while the complications caused by microvascular disease include retinopathy, renal disease and possibly polyneuropathy.

1.2 Platelets

Abnormalities of both in vivo and in vitro haemostasis have been extensively reported in diabetes (Jones et al, 1981; Colwell et al, 1983). Particularly, changes in platelet function have been described in cross-sectional studies of diabetic animals and patients, and cause-and-effect relationships have been postulated. The association of platelet and other haemostatic abnormalities which favour platelet deposition and thrombosis formation in diabetes can be interpreted in many ways. First, the abnormalities could contribute directly to the diabetic vascular disease; second, they could be totally unrelated; third, diabetic vascular disease could cause the defects; and fourth, they could be contributory to, and result from the vascular disease (Colwell et al, 1983).

The evidence that platelets have a role to play in the development of diabetic vascular disease is derived at least in part from the presence of platelet

abnormalities prior to histological evidence of vascular disease (Sagel et al, 1975; Halushka et al, 1981; Paulsen et al, 1981; Janka and Demmel, 1981). Further evidence is provided by the alteration in haemostatic factors with improvement of glycaemic control (Janka and Demmel, 1981; Juhan et al, 1982; Hiramatsu et al,1987), the detection of platelet microthrombi in experimental diabetes (Bloodworth and Moliter, 1965; Ishibashi et al, 1981) and reports of diminished rate of progression of diabetic vascular disease with the use of non-steroidal anti-inflammatory drugs (Powell and Field, 1964; Colwell et al, 1986; Donadio et al, 1988; The Damad Study Group, 1989).

1.3 Leucocytes

Many investigators have proposed a causal relationship between neutrophil accumulation at sites of inflammation and subsequent tissue changes, including local oedema, haemorrhage and thrombosis. As long ago as 1887, Metchnikoff postulated that during intracellular killing and digestion of phagocytosed bacteria, neutrophils may release substances extracellularly that damage vessel walls. However, the first convincing evidence for a critical role for neutrophils in vascular injury came from studies in the 1950's and 1960's using animals

depleted of circulating neutrophils by specific antibody or nitrogen mustard. Neutrophil depletion prevented immune-mediated vascular injury in the Arthus reaction, serum sickness, and nephritis (Cochrane and Janoff, 1975). Neutrophils have also been shown to be necessary for the increase in vascular permeability and tissue oedema induced by intradermal injection of chemotactic agents (Wedmore and Williams, 1981; Issekutz, 1981). Neutrophils have been particularly implicated in the development of respiratory distress syndrome (Cochrane et al, 1983) and in several models, acute lung injury has been attenuated by prior neutrophil depletion (Tate and Repine, 1983). It is reasonable to hypothesize that in certain circumstances, neutrophils adherent to the vessel wall release toxic products that damage endothelium or alter endothelial function. Three classes of mediators are particularly likely to participate in the pathogenesis of neutrophil-mediated vascular injury: granule enzymes, reactive oxygen metabolites, and products of membrane phospholipids (Harlan, 1985; Epstein, 1989).

Numerous abnormalities of neutrophil function have been described in diabetic patients. An elevated plasma glucose concentration inhibits neutrophil function and movement (Mowat and Baum, 1976; Molenaar et al, 1976), phagocytosis

(Bagdade et al, 1974), killing (Nolan et al, 1978), and oxidative metabolism (Nath et al, 1984). In addition, oxidative killing by neutrophils from patients with diabetes is inhibited by ß-hydroxybutyrate and galactose (Wilson et al, 1987), and this is considered to be secondary to induced changes in the polyol pathway due to an alteration in the redox potential.

1.4 Free radical activity

A free radical is an atom, ion or molecule with one or more unpaired electrons. This configuration leads to an increased reactivity with other molecules. The amount of reactivity depends on the ease with which a species can accept (is reduced) or donate (is oxidised) electrons.

During aerobic metabolism molecular oxygen (O_2) acts as the terminal electron acceptor and undergoes stepwise reduction to water. Oxygen is a bi-radical : it has two unpaired electrons in separate, parallel orbits both spinning in the same direction. Non-radical species have paired electrons spinning in opposite directions. One of these must spin invert before both can be accepted by oxygen. This process can occur, but limits the reactivity of molecular oxygen, which

preferentially accepts electrons one at a time (univalent reduction). The univalent reduction of oxygen produces the superoxide anion radical $O_2^{\cdot -}$

i.e. $O_2 + e^{\cdot -} \rightarrow O_2^{\cdot -}$ ($^{\cdot}$= unpaired electron and signifies a free radical).

The superoxide anion is comparatively unreactive. It has a very short half-life and its solubility in lipids is low. These properties limit its capacity to diffuse away from its site of generation and to affect cell membrane. In an ischaemic environment with acid pH, the anion will become pronated forming the more reactive perhydroxyl radical ($HO^{\cdot}_2$). As this radical is uncharged, and therefore more lipophilic, it can damage cell membranes.

In addition, because of its electronic configuration the superoxide anion can act both as a reducing agent and an oxidising agent, thereby allowing transition metal ions to act as catalysts in other free radical reactions. Further univalent reduction of the superoxide anion yields a non-radical species (O_2^{2-}), which exists at physiological pH in its pronated form, hydrogen peroxide (H_2O_2). This species can diffuse a considerable distance and can cause damage via its reduction to the hydroxyl radical (OH–). This reaction requires a transition metal ion such as ferrous ion as a catalyst.

$$H_2O_2 + Fe^{2+} \rightarrow OH^{\cdot} + OH^{-} + Fe^{3+}$$

The hydroxyl radical ($OH^{\cdot}$) is extremely reactive and has a very short half-life;

thus its damaging effects are very localised.

By virtue of their unpaired electrons, superoxide, hydrogen peroxide and hydroxyl radical react with virtually all cell components, especially sulphhydryl-containing amino acids and unsaturated fatty acids. The potential consequences of unimpeded free-radical reactions include the denaturation of proteins, oxidation and peroxidation of membrane lipids, generation of chemotactic factors, and impairment of collagen synthesis leading to loss of enzyme activity, disturbed membrane permeability, and increased inflammatory cell infiltration (Dormandy, 1983; McCord, 1985; Oberlay, 1988). As a result, free radical activity has been implicated in inflammation, aging, malignant change, alcohol-induced tissue damage (Dormandy, 1983; Fink et al, 1985) and recently vascular damage associated with myocardial ischaemia and infarction (Werns et al, 1986; Simpson et al, 1987). However, endogenous antioxidant defence mechanisms may play an important role in limiting oxidant-mediated vascular disease (Harlan et al, 1984), and alterations in their plasma and tissue concentrations are thought to reflect changes in free radical activity (McCord and Fridovich, 1977; Dormandy, 1983).

1.5 Aims

The pathogenesis of vascular disease in diabetes is poorly understood. Platelet abnormalities have been extensively investigated, and mainly through pharmacological manipulations, many abnormalities have been elucidated. Although both neutrophil elastase and free radical activity have been implicated in vascular disease, their roles in the development of diabetic microvascular disease have not been clearly assessed.

The work in this thesis has been concerned with platelet and leucocyte functional abnormalities, and also in changes in free radical activity in diabetic patients. The experimental section is divided into two main parts. The first part (section B) deals with platelet changes in insulin-dependent diabetic patients in relation to glycaemic control, proliferative retinopathy and improvement in glycaemic control. It also deals with changes in platelet parameters in non-insulin dependent diabetic patients at diagnosis, and assesses the effect of glycaemic control and oral hypoglycaemic therapy. The second section (section C) investigates changes in neutrophil elastase in relation to microvascular disease, and acute insulin-induced hypoglycaemia. In addition, this section is also

concerned with free radical activity in relation to microvascular disease in the form of retinopathy.

CHAPTER 2

PLATELET MORPHOLOGY AND AGGREGATION

PLATELET MORPHOLOGY AND AGGREGATION

2.1 Introduction

2.2 Platelet morphology

2.2.1 Peripheral zone

2.2.2 Sol-gel zone

2.2.3 Organelle zone

2.2.4 Membrane systems

2.3 Aggregation pathways

2.3.1 Primary aggregation

2.3.2 Secondary aggregation

2.3.3 Miscellaneous pathways

2.3.4 Platelet receptors

2.4 Second messengers

Introduction

Platelets have been extensively investigated during the past decade. The enormous amount of interest stems from the awareness of the roles that platelets play not only in haemostasis and thrombosis, but also, albeit less definitively, in atherogenesis and arterial spasm (Ross and Glomset, 1976; Ganda, 1980; Ross, 1986). Platelets form a human tissue that can be readily and repeatedly sampled. Furthermore, they can also act as a model for investigating stimulus-response coupling pathways that can be expected to have general implications for other cell types.

Platelets are very rapidly activated at sites of vascular injury and in fractions of a second they begin adhering to the damaged area. Receptors for fibrinogen, which is present in blood in large amounts, appear on the surface of the platelets and fibrinogen forms cell-cell bridges so that others attach to those already present to form the beginnings of a thrombus or platelet clump (Mustard et al, 1975). The platelets also undergo a shape change, becoming more spherical and putting out long pseudopodia. The contents of various classes of secretory granules are discharged by secretory exocytosis, and contents include serotonin (5HT) and adenosine-5'-

diphosphate (ADP) which can stimulate activation of other platelets.

2.2 Platelet Morphology

Platelets are anucleate cytoplasmic fragments originating from megakaryocytes in bone marrow. They circulate as biconvex discs (2-4um;7-8fl volume) and do not normally adhere to other platelets, endothelium or other blood cells. A platelet can be divided into four distinct regions: the peripheral zone, sol-gel zone, organelle zone and membrane systems.

2.2.1 Peripheral zone

The peripheral zone constitutes the platelet wall and invaginates extensively into the interior of the cell to form an open canalicular system. It maintains platelet integrity, provides receptor sites for various stimulating or inhibiting agents, is responsible for platelet immunological specificity and mediates platelet-platelet and platelet-surface interactions. It is composed of three morphological domains: the exterior coat ("glycocolyx"), the unit membrane, and the submembrane region. The surface glycoproteins contain sialic acid molecules contributing to a large

negative surface charge which prevents, by electrostatic repulsion, platelets from sticking to each other and to normal intact endothelium (Görög et al, 1982). The unit membrane consists mainly of a phospholipid bilayer and plays an important role in accelerating blood coagulation. Other important components of the platelet unit membrane include the membrane endoenzymes particularly the enzymes involved in membrane transport and cyclic AMP (cAMP) metabolism. The submembrane region contains a system of filamentous elements and may be functionally involved in maintaining the platelet discoid shape, pseudopod formation and clot retraction.

2.2.2 Sol-gel zone

The sol-gel zone comprises the viscous matrix inside the platelet and is composed of three different fibre systems: submembrane filaments, microtubules and microfilaments. This zone is also functionally involved in maintaining platelet shape.

2.2.3 Organelle zone

Several different types of organelle can be detected in the cytoplasm of platelets.

They have a random distribution in non-activated platelets but are centralised upon activation. The electron dense bodies are intensively opaque and contain a non-metabolic ("storage") pool of calcium, 5HT, adenosine- 5'-diphosphate and triphosphate (ADP and ATP), all of which are secreted during the release reaction. The non-dense granules are the most numerous platelet organelles. Cell fractionation techniques and cytochemical studies have revealed that they can be subdivided into alpha-granules, lysosomes and peroxisomes (Bentfeld-Barker and Bainton, 1982). The alpha-granules are storage sites containing a number of proteins, including platelet-specific ß-thromboglobulin (ßTG) and platelet factor 4 (PF4). These are both tetramers of basic proteins and the plasma levels are thought to reflect in vivo platelet activation (Ludlam et al, 1975; Kaplan and Owen, 1981). Lysosomes contain several acid hydrolases and are released into the external milieu by high concentrations of aggregating agents. Their role may be in the ultimate disposal of platelet debris during the process of wound healing following vessel trauma.

2.2.4 Membrane systems

The open canalicular system provides an anastomising network spreading

throughout the cytoplasm giving a greatly enlarged platelet surface area and making plasma-borne substances much more accessible to the inner part of platelets. Externalisation of the internalised platelet membranes appears to take place during platelet shape change (Frojmovic and Milton, 1982), possibly resulting in the exposure of functional sites. The dense tubular system is derived from the smooth endoplasmic reticulum and constitutes a continuous network of narrow canaliculi. It is the major site for calcium which can be mobilised to regulate contractile events. In addition, prostaglandin metabolism also appears to be localised in the dense tubular system.

2.3 Aggregation pathways

A process of activation can be inferred to be a pre- requisite for platelet adhesion and aggregation, from the non-reactivity of normal circulating platelets. It is probable that no single agent is responsible for the initiation of platelet aggregation "in vivo". The initiation and perhaps growth of thrombin from platelet aggregation is influenced by products of the arachidonic acid pathway (Figure 2.1). Thromboxane $(TX)A_2$ is a metabolite of arachidonic acid and is synthesised within, and released from the platelet. It is a potent stimulator of platelet aggregation and

constrictor of blood vessels (Hamburg et al, 1975; Moncada and Vane, 1978). Prostacyclin (PGI_2), another arachidonic acid metabolite is produced by endothelial cells and has opposing actions to TXA_2, whereby, it inhibits aggregation and dilates blood vessels. A balance locally between PGI_2 and TXA_2 may therefore prevent thrombosis and haemostatic plug formation (Moncado and Vane, 1978; MacIntyre et al, 1978). Futhermore it has been suggested that an imbalance in the PGI_2-TXA_2 system may contribute to certain pathological conditions including arterial thrombosis (Lagarde and Dechavanne, 1977), and thrombocytopenic purpura (Remuzzi et al,1980). Prostacyclin synthetase, the enzyme which converts prostaglandin endoperoxides (PGG_2 and PGH_2) to PGI_2 is most highly concentrated in the intimal surface of the blood vessel, and its concentration progressively decreases towards the adventitial surface (Moncada et al, 1977; Moncada and Vane, 1978). Damaged endothelium may have a reduced capacity for PGI_2 production, thus allowing platelet deposition to occur more readily. A fall in PGI_2 production at the site of the damaged vessel may be a contributory factor to the initiation of thrombus formation.

2.3.1 Primary aggregation

Primary aggregation is a reversible process and results from the direct effect of

agonists at the platelet surface, and it is not effected by inhibitors of the cyclo-oxygenase pathway. Secondary aggregation occurs as a consequence of the endogenous synthesis of TXA_2 and/or the release reaction. If the stimulus to produce primary aggregation is not sufficient to induce thromboxane synthesis or the release reaction, aggregation spontaneously reverses and the platelets retain their discoid shape (Mustard et al, 1975). Primary aggregation also reflects the exposure of fibrinogen receptors on the platelet surface, and the interaction with fibrinogen subsequently enhances cell-cell contact (Mustard et al,1975). This response requires a lower cytosolic calcium concentration than that necessary to induce secondary wave aggregation and may involve a pool of bound calcium ions (Charo et al, 1976; Le Breton and Dinnerstein, 1977). It has also been suggested that extracellular calcium ions are essential for the primary response since in the presence of the chelating agent EDTA, primary aggregation is suppressed. In the presence of physiological calcium concentrations, ADP induces a readily reversible platelet aggregatory response. However, in a low calcium concentration medium such as citrated plasma, high concentrations of ADP (1-2μM) induces two phases of aggregation in human platelets; the second phase is associated with activation of the arachidonic acid pathway and the release reaction (Mustard et al,1975). Human platelets prepared with other anticoagulants such as hirudin and exposed to physiological calcium concentration only demonstrate primary aggregation waves

(Kinlough-Rathbone et al, 1983).

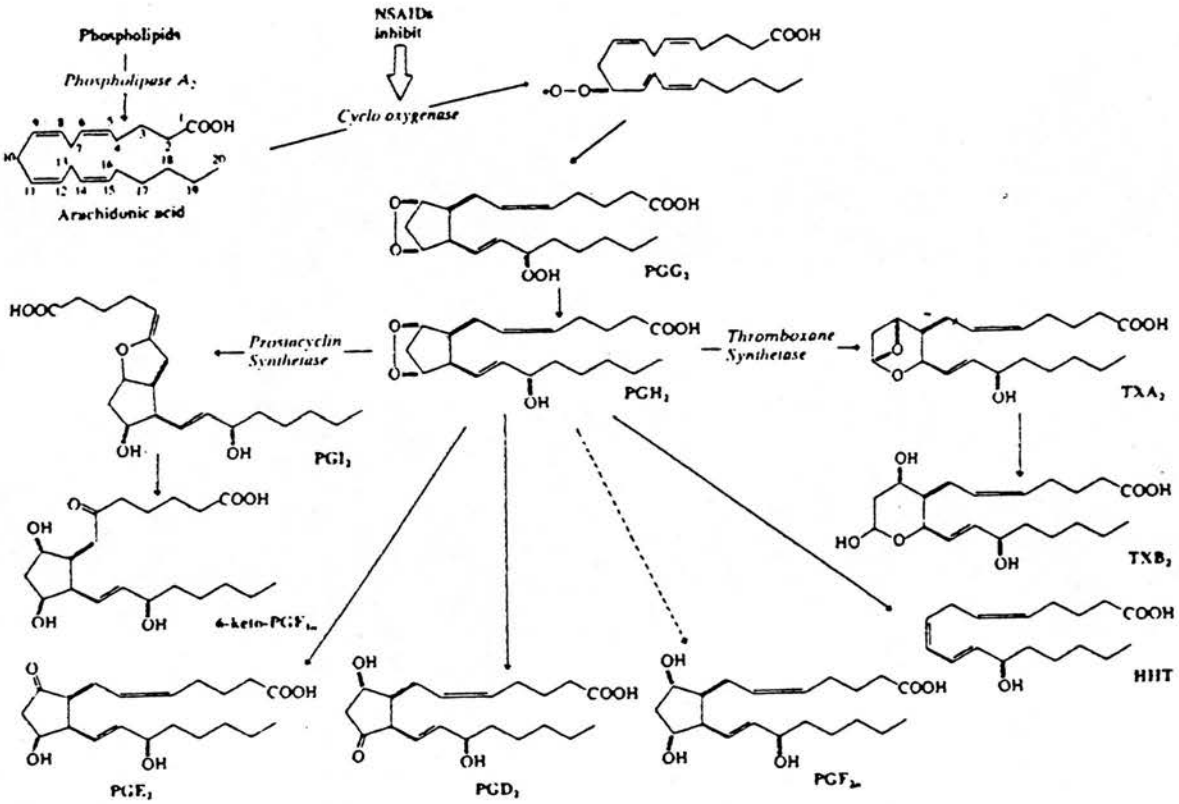
2.3.2 Secondary aggregation

Although primary aggregation is of considerable pharmacological interest, it may be an artifact of agonist-induced platelet aggregation in vitro (Armstrong et al, 1983; Kinlough-Rathbone et al, 1983). However, as indicated already, secondary aggregation occurs as a consequence of the endogenous TXA_2 and/or the release reaction synthesis, and during this reaction the contents of platelet storage vesicles are released from platelets. Agents that can induce secondary wave aggregation include thrombin, collagen, PAF and TXA_2. They do so through one or both of the following:

A) by activation of the phospholipase enzymes which cleave arachidonic acid from membrane phospholipids. Arachidonic acid is then converted to the prostaglandin endoperoxides (PGG_2 and PGH_2) and TXA_2

B) by directly triggering the release of ADP and 5HT from platelet dense granules.

Figure 2.1



The biosynthesis of prostaglandins and thromboxanes from arachidonic acid

Movement of calcium across platelet membranes appears to be important as the release reaction can be induced by calcium ionophores (Feinman and Detwiler, 1974) and inhibited by calcium antagonists (Charo et al, 1976). Calcium movement into the cytoplasm probably exerts an effect on the platelet microtubules which move towards the centre of the cell in a contractile wave (White et al, 1972). In addition, activation of the calcium-dependent platelet actin-myosin complex is also thought to be involved (Haslam and Lynham, 1977; Daniel et al, 1981. The actin-myosin complex has also been postulated to be involved in the vesicular fusion of dense bodies with the plasma membrane, whereby an opening is formed through which the contents are expelled (Daniel et al, 1981). Protein phosphorylation reaches a maximum before completion of secondary aggregation and is followed by slow dephosphorylation (Feinstein et al, 1983). No increase in phosphorylation is observed when aggregation without secretion occurs, and it therefore seems likely that phosphorylation is a pre-requisite to the initiation of secretion.

The formation of endogenous TXA_2 during secondary wave aggregation occurs due to activation of phospholipase A_2 which induces arachidonic acid release from membrane phospholipids (Hamburg et al, 1975). The role of endogenous TXA_2

was primarily elucidated as a result of studies on the mode of action of non-steroidal anti-inflammatory drugs (NSAID) such as aspirin and indomethacin, which inhibit prostaglandin synthetase (cyclo-oxygenase) enzyme (Vane, 1971; Moncada and Vane, 1978).

ADP, 5HT and TXA_2 potentiate aggregation by either recruiting other platelets, or in the case of TXA_2, trigger further release and it is these events which induce the second phase of aggregation. In addition, ADP,5HT and TXA2 may act synergistically with weak aggregating agents such as adrenaline (Kinlough-Rathbone et al, 1977).

2.3.3 Miscellaneous pathways

A third pathway of aggregation which is independent of both TXA_2 and ADP has also been postulated. In the presence of NSAIDs or ADP removing enzymes (creatinine phosphate or creatinine and collagen phosphokinase; CP/CPK) thrombin induced aggregation is only partially inhibited, indicating the involvement of another mechanism (Kinlough-Rathbone et al, 1977; Cazenave et al, 1979). It has been suggested that a phospholipid, 1-0-alkyl-2-acetyl-glyceryl-3-phosphorylcholine (Platelet Activating Factor,PAF) may be the mediator of this

third pathway (Vergaftig et al, 1981). It has been demonstrated that as a consequence of calcium ionophore stimulation, radioactive acetate incorporated in platelet membrane phospholipids is subsequently present in PAF. This would suggest that PAF may play a role as an intracellular mediator of aggregation (Chap et al, 1981). Furthermore, platelets desensitized to PAF lose their ability to respond to thrombin. However evidence has been put forward, against PAF as mediator of the third pathway of aggregation. It has been demonstrated that the NSAID, acetylsalicylic acid together with CP/CPK completely inhibit PAF-induced aggregation and secretion (Kloprogge et al, 1983). In addition, both desensitisation to PAF (Hallam et al, 1984), and congenital absence of PAF synthesis does not significantly reduce responsive to thrombin or collagen. Therefore PAF possibly plays only a minor role, if any at all, in platelet activation induced via a third pathway.

In addition to the three mechanisms described above which require the presence of agonists, platelets can also aggregate as a result of close cell-cell contact, whereby activation of the arachidonic acid pathway with the subsequent formation of TXA_2 can trigger the release reaction and promote secondary aggregation. This type of aggregation is blocked by NSAIDs, and the release reaction is secondary to close

platelet contact and aggregation (Massini and Zuscher, 1974).

2.3.4 Platelet receptors

Irrespective of the precise mechanism underlying the platelet response, whether it is shape change, primary or secondary wave aggregation, activation of platelets is initiated by agonist interaction at the surface membrane usually at discrete receptor sites. Surface receptors have been identified for ADP (Nachman and Ferris, 1974), thrombin (Detwiler and Feinman, 1973; Tollefsen and Majerus,1976), adrenaline (Bydgeman and Johnsen, 1969), 5HT and certain prostaglandins and TXA_2 (Le Breton et al, 1979; Nicolaou et al, 1979). In addition to their action at discrete receptor sites, 5HT and adrenaline, can be actively taken up into platelets, a phenomenon not associated with aggregation.

Most of the compounds mentioned above induce only aggregation while metabolites of arachidonic acid can either induce or inhibit aggregation. The prostaglandin endoperoxides, PGG_2/PGH_2 and TXA_2 fall into the former category, acting at a "TXA_2/endoperoxide receptor". There is controversy as to the true biological activities of the endoperoxides. For example, it has been reported that thromboxane synthetase inhibitors virtually abolish arachidonic acid aggregatory activity

(Fitzpatrick and Gorman, 1977), and that the concentrations of endoperoxides in platelets following arachidonic acid induced aggregation are 100 times lower than would be required for aggregation. In contrast, it has been demonstrated that the addition of exogenous PGG_2 to platelets "in vitro" induces very rapid aggregation and release (Claesson and Malsten, 1977). Exogenous PGH_2 is degraded in platelet-rich-plasma predominately to PGD_2 and PGE_2 with less than 1% conversion to TXA_2 (Oelz et al, 1977). Therefore direct action of PGG_2 or PGH_2 may be masked by their rapid transformation to PGD_2 which is a potent inhibitor of aggregation (Oelz et al, 1977). This may explain why low levels of endoperoxides are detected in some studies and why the thromboxane synthetase inhibitor, Dazoxiben, may block aggregation (Heptinstall and Fox, 1983). In addition, other workers have suggested that arachidonic acid conversion to TXA_2 is not essential for aggregation (Needleman et al, 1976) supporting the concept that endoperoxides themselves are capable of inducing aggregation.

However, it is generally assumed that the endoperoxides and TXA_2 act on a common receptor; the greater potency of TXA_2 has lead to the designation of "thromboxane receptor". From a chemical viewpoint the majority of the TXA_2 mimetics studied are in fact endoperoxide-like in structure (Corey et al, 1977; Bundy, 1975). Furthermore, receptor antagonists are capable of blocking the aggregatory effects

of both PGH_2 and TXA_2 suggesting that these antagonists are acting on a common receptor (Armstrong et al, 1983; Armstrong et al, 1986).

Platelets also contain receptor systems responsive to prostaglandins E_1, I_2 and D_2. Occupation of these receptors leads to an activation of adenylate cyclase with subsequent inhibition of platelet function due to elevations in cyclic AMP. Two distinct receptors for PGD_2 and PGI_2 have been identified (MacIntyre and Gordon, 1977) with PGE_1 acting on PGI_2 receptors. Recently, PGE_1 has been reported to inhibit platelet aggregation independent of cyclic AMP involvement (Sinha and Colman, 1985). The precise mechanism underlying this inhibition remains to be elucidated.

2.4 Second messengers

Essentially, platelet responses are evoked by the interaction of agonists at specific receptors and it is appreciated that such responses are a sequelae of controlled intracellular reactions mediated by second messengers. Phospholipase A_2, a membrane bound enzyme is stimulated by many agonists, and activation of this enzyme releases arachidonic acid and possibly PAF acether from membrane

phospholipids which initiates intracellular platelet mechanisms including cyclic AMP changes and Ca^{2+} fluxes.

Platelet activation by thrombin and ADP is coupled with phosphoinositide turnover, with information of intraplatelet second messengers 1,2-diacylgylcerol and inisitol 1,4,5-triphosphate (IP_3) from hydrolysis of phosphatidylinisitol-4-5- biphosphate (Vickers et al, 1982; Berridge 1984). Subsequent formation of arachidonic acid from diacylglycerol, and by the activation of phospholipase A_2, yields proaggregatory prostaglandin metabolites, such as TXA_2 (Vickers et al, 1982; Berridge 1984).

IP_3 has been postulated to act as an intracellular messenger triggering calcium fluxes. In human platelets stimulated with the endoperoxide analogues (U44069 and U46619), a rapid formation of diacylglycerol and phosphatidic acid has been reported (Pollock et al, 1984), a process which was found to be insensitive to non-steroidal anti-inflammatory drugs.

Thrombin can also induce aggregation through activation of phospholipase C with resultant formation of IP_3, despite the presence of aspirin (Di Minno et al,

1986). However the calcium ionophore (U44069) does not activate phospholipase C in the presence of aspirin, apyrase or CP/CPK, and it seems likely that phospholipase C is activated by agonists acting through receptors (Seiss et al, 1984). Alternatively, the calcium ionophore activates phospholipase C indirectly by stimulating phospholipase A_2 (which is sensitive to a Ca^{2+} fluxes) which releases arachidonic acid, and it is the prostaglandins and TXA_2 which induces phospholipase C to generate polyphosphoinositides (Seiss and Lapetina, 1988).

Cyclic AMP is a further second messenger, and it is generally accepted that inhibiting agents depress platelet responsiveness as a consequence of an increase in the level of cAMP (Haslan et al, 1978). It is generated in platelets by stimulation of adenylate cyclase and may in part act by increasing intraplatelet calcium sequestration (Owen et al, 1980). Cyclic AMP also inhibits intracellular release of calcium and influx from the surrounding media (Feinstein et al, 1983), thus reducing protein phosphorylation due to a restriction in the amount of cytoplasmic calcium available to these enzymes (Kawahara et al, 1980). Futhermore the lowering of cytoplasmic calcium by cyclic AMP stimulators closely corresponds to its ability to reverse thrombin induced aggregation, protein phosphorylation and cytoskeleton assembly (Feinstein et al, 1983). It has been postulated that a cAMP dependent protein-kinase is involved in calcium ion reuptake, and the

phosphorylation of proteins which are involved in the active pumping of calcium ions from the cytoplasm to intracellular stores. Studies have also suggested that increases in cAMP may depress phospholipase C activity in intact platelets and in this situation cAMP would be modulating the response of calcium indirectly by exerting control at the level of diacylglycerol (Rittenhouse-Simmons, 1979). In addition to stimulating active sequestration of calcium, and the inhibition of the phospholipase C enzyme, several groups have reported that an elevation in cAMP inhibits both arachadonate liberation from phospholipids (Gerrard et al, 1977; Lapetina et al, 1977) and possibly the cyclo-oxygenase enzyme (Malmsten et al, 1976): both of these enzymatic processes being calcium dependent.

The ability of cAMP therefore to lower cytosolic calcium, highlights an important role for cAMP, both as a modulator of the initiation of platelet activation and in reversal of the activation process following stimulation.

It is clear from this chapter that platelets are complex entities. They function as a result of the interactions of many biochemical processes occurring at the surface membrane and the intracellular organelles, and only through pharmacological manipulations have many of the underlying mechanisms been elucidated.

CHAPTER 3

NEUTROPHIL ELASTASE AND FREE RADICAL ACTIVITY

NEUTROPHIL ELASTASE AND FREE RADICAL ACTIVITY

PART A

3.1 Introduction

3.2 Neutrophil granules

3.3 Human neutrophil elastase

PART B

3.4 Introduction

3.5 Phagocytosis and bactericidal killing

3.6 Plasma free radical activity

PART A

3.1 Introduction

The major function of neutrophil granulocytes is to prevent or retard the intrusion of infectious agents and other foreign material into the host environment. This is accompanied by phagocytosis and digestion of the material. Neutrophils also release various substances into their environment and thus also have a secretory function.

Neutrophil production in normal adult man appears to take place only in the bone marrow. The life cycle of the neutrophil can be divided conveniently into bone marrow, blood and tissue phases. It is assumed that cells move through the system in a more or less orderly manner as if in a pipeline: this view is supported by the progressive movement of isotopic tracers (Maloney and Patt, 1958) and azurophilic granules (Wetzel, 1970) through the system.

3.2 Neutrophil granules

There are two granule types in neutrophils; one comprises about one-third of all neutrophil granules, serves primarily an intracellular role, and has the characteristics of lysosymes. The other granules, which are more abundant, are readily accessible to extracellular release, and in many respects are like granules secreted by secretary organs. The maturation characteristics of neutrophils clarifies the two types of granules. Neutrophil development occurs in two phases: the mitotic and post-mitotic phase both last around a week. The first granules appear in the promyelocytic stage. They are packaged and released from the inner concave surface of the Golgi apparatus, and stain azurophilic in the bone marrow. They appear first, and are therefore also called the "primary" granules. Later in maturation these granules stop being synthesised and are distributed uniformly in daughter cells so that in mature neutrophils there are relatively few primary granules. By this stage, a second granule predominates which is formed and released from the outer convex surface of the Golgi apparatus. Since these granules occur second in granule maturation, they are called "secondary" granules and because they also have unique qualities, they are also called "specific" granules. The specific granules are particularly accessible for fusion with the plasma

Table 3.1

Subcellular localisation of enzymes and other constituents released by human neutrophils

Class of constituents	"Primary" (Azurophilic) Granules	"Secondary" (Specific) Granules
Microbial enzymes	Myeloperoxidase Lysozyme	Lysozyme
Neutral serine proteinases	Elastase Cathepsin G Proteinase 3	
Metalloproteinase	Collagenase	*Collagenase
Acid hydrolases	N-acetyl-ß gluclsaminidase Cathepsin B Cathespin D ß Glucuronidase ß Glycerphosphatase Mannidase	
Other		Lactoferrin Vitamin B12 binding proteins Cytochrome 6

*Secondary granule collagenase is released as a latent enzyme

membrane (Bainton, 1977) with release of their contents to the extracellular environment. These granules defer a secretory function to neutrophils which, unlike more classical organs, are dispersed throughout the body.

3.3 Human neutrophil elastase

Human neutrophil elastase is a serine proteinase which occurs in neutrophils and myeloid precursors, and is largely concentrated in the azurophilic granules of granulocytes (Janoff, 1985). It is a single-chain polypeptide with a strongly basic isoelectric pH, and is discharged into plasma when the cell encounters objects to be phagocytosed or undergoes death. Turnover of connective tissue macromolecules by elastase secreted by histiocytes (tissue macrophages), smooth muscle cells, and fibroblasts appears to reflect a physiological process. However, hydrolysis of matrix molecules by neutrophil elastase probably reflects a pathological process (Janoff, 1985). In the latter situation, a wide variety of structurally important proteins and glycoproteins can be attacked by the neutrophil elastase; elastin, being an obvious target (Janoff and Scherer, 1968). Neutrophil elastase can also cleave core proteins off proteoglycan molecules in the

connective tissue ground substance (Kaiser et al, 1976). This can interfere with water entrapment by proteoglycan complexes, and in joint cartilage, spoil mechanical functions such as resistance to compressive forces. Other important structural targets include types (iii) and (iv) collagen; type (iii) collagen being a major supporting component of blood vessels, lung connective tissue and gut. Type (iv) collagen is important maintaining the structural integrity of epithelial and endothelial basement membranes. Fibronectin, a major cell-adhesion molecule, is another structural macromolecule susceptible to degradation (McDonald and Kelly, 1980). Although it was once thought that the connective tissue matrix acted only as an extracellular scaffold, it has been clearly shown to serve as an interactive substratum that regulates the shape, migration, growth, and differentiation of cells. In addition, it also has an important role in the orderly repair of damaged tissues (Vracko, 1974). Thus, if neutrophils mediate the dissolution of extracellular matrix, then it is probable that this leads to functional and structural damage.

In addition to connective tissue architectural components, many plasma proteins can be hydrolyzed by neutrophil elastase. Potential substrates include immunoglobulins, clotting factors (including fibrinogen) and complement proteins

(Janoff, 1985). Inactivation or activation of plasma cascades may have important local and systemic consequences (Plow, 1982). Formed blood elements can also be effected by the enzyme with both lymphocyte activation and platelet aggregation having been described (Havemann and Gramse, 1984).

Neutrophil elastase activity is normally restricted to the phagocytic vacoule and the immediate environment of the cell, and usually involves the presence of circulating and locally produced elastase inhibitors. Circulating inhibitors include alpha-1-proteinase inhibitor (formerly known as alpha-1-antitrypsin) and alpha-2-macroglobulin. Locally produced regulators of importance are "antileukoprotease" (Fritz et al, 1978) which are low molecular weight, acid-stable inhibitors present in bronchial, cervical and seminal plasma. Alpha-1-proteinase inhibitor appears to be the most important regulator of neutrophil elastase activity, and functions by complexing with elastase after being cleaved (Brower and Harpel, 1983). It is a glycoprotein synthesised in the liver, has a molecular weight of 52 kd, and individuals lacking normal levels in their circulation and bronchoalveolar fluids are at risk of developing chronic obstructive airways disease (Larrell and Erikson, 1963).

In vivo, the calculated half-life of active elastase is only about 0.6 msec; by 3

msec all activity should be inhibited (Travis and Salvesen, 1983). Given the effectiveness and multiplicity of the inhibitors that comprise the anti-elastase shield, it might seem reasonable to assume that neutrophil elastase would not have the opportunity to mediate extracellular tissue damage. However, there is considerable evidence that neutrophils not only circumvent the entire anti-proteinase shield, but also are able to use their discharged elastase to attack and destroy host tissues. For example, it has been demonstrated that isolates of purulent fluids recovered from sites of inflammation contain free neutrophil enzymes capable of degrading a variety of native and denatured proteins at neutral pH (Opie 1922; Weiss and Regiani, 1984). Alpha-1-proteinase inhibitor contains methionine at its reactive centre (position 358) which is sensitive to oxidation by either chemical reagents or triggered neutrophils. It seems likely that this causes a significant decrease (approximately 2000 fold) in the rate of association between neutrophil elastase and the modified anti-1-proteinase inhibitor (Travis and Salvesen, 1963; Janoff, 1985), resulting in increased elastase activity.

PART B

3.4 Introduction

Neutrophils are motile, and are therefore free to migrate into sites of inflammation. Once in the area of inflammation they come into contact with foreign material, engulf it, and subject it to the bactericidal and digestive enzymes that they contain. Endocytosis is the process by which material is taken into a cell enclosed within pieces of plasma membrane without the material at any time occurring free in the cytoplasm of the cell (Cohn and Hirsch,1960; de Duve and Watliaux, 1966). Endocytosis is further divided into pinocytosis (drinking by cells) and phagocytosis (eating by cells). Phagocytosis can usually be seen by light microscopy, whereas pinocytosis cannot because it involves ingestion of much smaller particles. Both processes involve invagination of the cell membrane and the formation of vesicles or vacoules (phagasomes). Although neutrophils ingest foreign material, they rarely ingest autologous cells (Vaughn, 1965)

3.5 Phagocytosis and bacterial killing

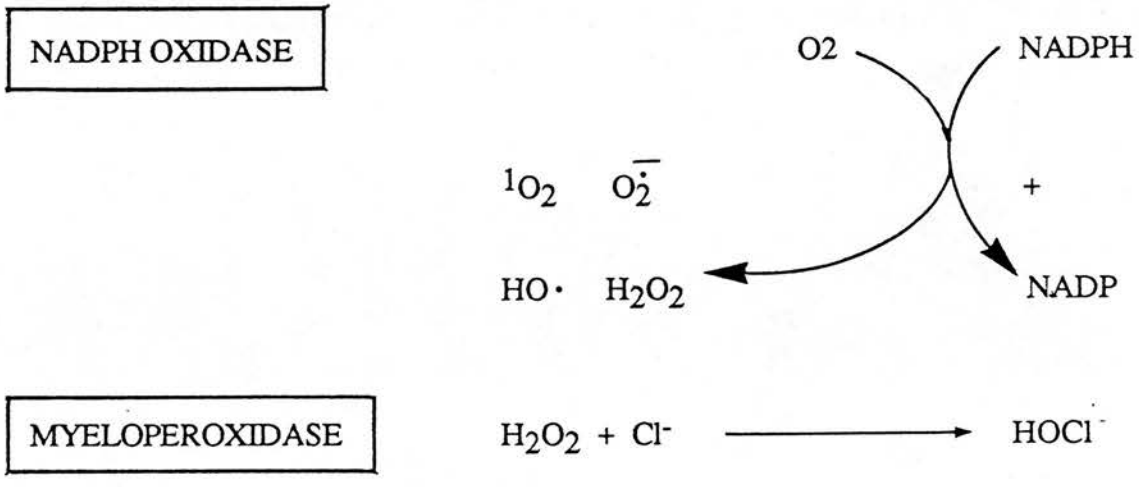
The ingestion of particles by phagocytic cells requires the expenditure of energy (Karnovsky, 1968) and is accompanied or immediately followed by a variety of complex biochemical events. Although neutrophils contain a cytochrome system (Cline, 1965) and may use both aerobic and anaerobic mechanisms to produce energy, they utilise anaerobic glycolysis to support phagocytosis (Shara and Karnovsky,1959). During ingestion the particle is surrounded by pseudopodia and the cell membrane invaginates to enclose and internalise it (Goodman and Moore, 1956). This phase is rapidly followed by a fusion of lysosome granules into the phagocytic vacoule (Cohn and Hirsch, 1960). In neutrophils, the content of specific granules are released into the phagasomes first (within 30 seconds), and the contents of primary granules are found in the phagasome later (1-3 minutes).

After fusion, bacteria are usually killed quickly and the phagocytosed material is digested. Two principally different mechanisms appear to be responsible for the bacterial killing by neutrophils. One is dependent upon the uptake of oxygen and the formation of toxic oxygen species; the other is dependent on the action of pre-formed bactericidal granule proteins such as cathepsin G, lactoferrin, lysozyme,

myeloperoxidase and permeability-increasing protein (Epstein, 1989). In addition, elastase, although not bactericidal by itself, may act by potentiating the bactericidal properties of other components (Havemann and Gramse, 1984).

The "respiratory burst" that occurs during bactericidal killing refers to a series of metabolic events that take place when neutrophils are appropriately stimulated, namely, an increase in oxygen consumption, the production of superoxide, activated oxygen species, and increase in glucose oxidation via the hexose-monophosphate shunt (Barbior, 1978). These events depend on the activity of an oxidase which catalyzes the one electron reduction of oxygen to superoxide, using an electron donor such as nicotinamide adenine dinucleotide phosphate (NADPH) (Barbior, 1978). In the presence of iron salts, particularly neutrophil lactoferrin, hydrogen peroxide can be converted to hydroxyl radicals (Ambruso and Johnston, 1981). Neutrophil myeloperoxidase, which is present in high concentration in the azurophilic granules of neutrophils is released into the phagasome during granule-phagasome fusion (Klebanoff, 1970). Myeloperoxidase, in the presence of hydrogen peroxide and a halide, is responsible for further catalyzing the formation of additional oxidants such as hypochlorous acid and free chlorine (Figure 3.1). This forms a potent anti-microbial system against bacteria, fungi, viruses and

Figure 3.1



Proposed mechanism for neutrophil-derived free radicals and toxid oxygen metabolites. Membrane-associated NADPH oxidase reduces molecular oxygen to form superoxide anion ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$) and hydroxyl radical HO·. The granule-associated myeloperoxidase catalyses the conversion of hydrogen peroxide (H_2O_2) to the toxic metabolite hypocholorous acid (HOCl).

mycoplasma; this system is also toxic to other cells including tumour cells.

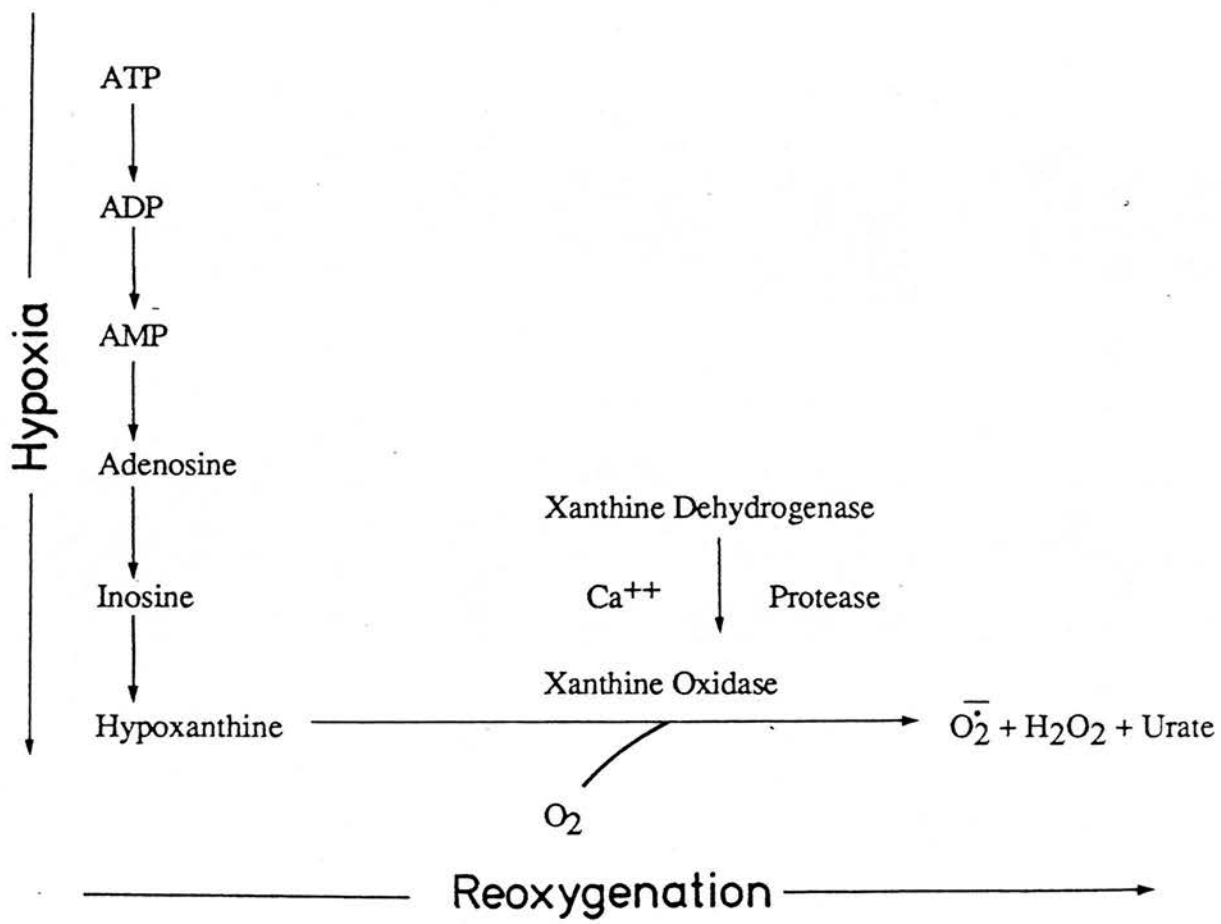
As has already been indicated, human neutrophil polymorphonuclear leucocytes provide an effective defence against bacteria and fungal infection. However, they may also have an important role in the pathogenesis of tissue damage in certain non-infective diseases (Malech and Gallin, 1987). Mild to moderate abnormalities of neutrophil function have been reported in many acquired and congenital diseases (White and Gallin, 1986; Rotrosen and Gallin, 1987) and in most of the disorders, the biochemical and morphological basis are still unclear. Neutrophil elastase and free radical activity have been implicated in the development of myocardial ischaemia and infarction (Werns et al, 1986; Simpson et al, 1987). In addition, there is synergism between neutrophil elastase and oxidizing systems; activated oxygen species and activated halides can not only damage tissue themselves, but can also inactivate alpha-1-proteinase inhibitor (Janoff, 1985). Elastase-mediated injury to airways has been implicated as a pathogenic factor in cystic fibrosis (Jackson et al, 1984; Suter et al, 1984); increased levels of active neutrophil elastase have been reported in bronchoalveolar washings from patients acutely ill with respiratory distress syndrome (Lee et al, 1981; McGuire et al, 1982; Till et al, 1982), and as has already been discussed, neutrophil, monocyte and

macrophage elastases have also been implicated in the development of emphysema (Janoff, 1985).

3.6 Plasma free radical activity

It seems likely that free radical activity in plasma is derived from a number of sources. Neutrophil NADPH oxidase and myeloperoxidase produce toxic oxygen metabolites, and are possibly the major source. In addition at least three other sites may contribute. Vascular endothelium, particularly during periods of ischaemia and reperfusion, is a further source (Werns et al, 1986; Simpson et al, 1987). The cytosolic enzyme xanthine oxidase is normally present in a dehydrogenase form. During ischaemia, the dehydrogenase form is converted to the true oxidase form, which uses oxygen, instead of nicotinamide adenine, as an electron acceptor. On reperfusion of ischaemia tissue, the delivered oxygen can be reduced by this system, producing superoxide and hydrogen peroxide (McCord, 1985; Chambers et al, 1985). (Figure 3.2). Free radicals are also synthesised during arachidonic metabolism (Yamato, 1983; Kontos, 1987); activated phospholipids release arachidonic acid which accelerates synthesis of free radical intermediates by the cyclooxygenase and lipoxygenase pathways. In addition, free

Figure 3.2



Proposed mechanism for free radical injury induced by anoxia-reoxygenation (ATP = adenosine triphosphate; ADP = adenosine diphosphate; AMP = adenosine monophosphate; O_2^- = superoxide radical; H_2O_2 = hydrogen peroxide).

radicals may be derived from the hepatic microsomal system, and indeed hepatocytes from ketotic rats produce increased lipid peroxidation which can be suppressed in vitro by insulin (Kosugi et al, 1984).

As discussed elsewhere, unless free radicals are rapidly eliminated, they are capable of producing cellular and tissue damage (Fridovich, 1978). The levels of the activated oxygen species are controlled by various cellular defence mechanisms consisting of enzymatic (superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic (glutathione and vitamin E) scavenger components (Simmons, 1984; Halliwell and Gutteridge, 1984). There is increasing evidence that, in certain pathological states, along with increased free radical activity, the ineffective scavenging of such activated oxygen species may play a role in the development of tissue injury (Halliwell and Gutteridge, 1984; Dormandy, 1983).

Free radicals have a lifespan of microseconds and their concentration at any site at any moment is miniscule. Therefore the evidence for their existence is mostly indirect. Superoxide dismutase, which uses the free radical anion superoxide as a substrate (McCord and Fridovich, 1977), has been the chief enzyme and

scavenging system investigated. The reduced concentration of superoxide dismutase and other scavenging enzyme systems, is usually interpreted as reflecting increased free radical activity (McCord and Fridovich, 1977; Dormandy, 1983). Recently, as discussed in Chapter 10, it has been demonstrated that the free radical mediated oxidation product of linoleic acid, PL-9,11-LA', is a good marker for free radical activity (Cawood et al, 1983; Iversen et al, 1984).

CHAPTER 4

PLATELET DENSITY ANALYSIS AND INTRAPLATELET CONTENT IN INSULIN-DEPENDENT DIABETIC PATIENTS

PLATELET DENSITY ANALYSIS AND INTRAPLATELET GRANULE CONTENT IN INSULIN-DEPENDENT DIABETIC PATIENTS

4.1 Introduction

4.2 Subjects

4.3 Methods

4.3.1 Platelet density analysis

4.3.2 Measurement of intraplatelet nucleotides

4.3.3 Measurement of intraplatelet ß-thromboglobulin

4.3.4 Glycosylated haemoglobin

4.3.5 Statistical analysis

4.4 Results

4.5 Discussion

4.1 Introduction

It is recognised that circulating platelets are heterogeneous with respect to function, volume and density; however the exact mechanism by which this heterogeneity is created remains controversial. It may arise in the bone marrow during the production of platelets by megakaryocytes (Thomson et al, 1983) or, alternatively, within the circulation after liberation from the marrow. The latter possibility has aroused much controversy; some studies report an increase in density as platelets age in the circulation (Mezzano et al, 1981; Boneu et al, 1982) whereas the more traditional view is that they become less dense during their lifespan (Corash and Shafer, 1982, Rand et al, 1983). Watson and Ludlam (1986) investigated platelet-density and survival in normal and post-splenectomized subjects, and concluded that high density platelets were preferentially retained in the spleen and have a more prolonged survival than those of lower density.

Platelets contain several classes of secretory granules, including lysosomes, electron-dense granules (containing ADP, serotonin (5-HT), and Ca^{2+}) and alpha-granules (containing ß thromboglobulin (ßTG), fibrinogen, platelet factor

4, factor V (proaccelerin), and factor VIII), and during aggregation these are released into the surrounding plasma. Cieslar et al (1979) demonstrated that platelet de-granulation, induced by thrombin, was accompanied by a decrease in platelet density, and it has been proposed that the alpha-granule content is the major determinant of platelet density in vivo (Vicic and Weiss, 1983; van Oost et al, 1984). After a study of the relationship between platelet-density distribution and granule-marker levels in acquired storage-pool disease, Boneu et al (1983) suggested that platelet-density analysis might be a useful screening test to detect activated platelets in the circulation. In this study, the density profiles of platelets from insulin-dependent patients with differing prevailing metabolic control have been investigated, and related to their intraplatelet granule content.

4.2 Subjects

Thirty-two insulin-dependent diabetic patients (18 males, 14 females) aged 29.4 ± 3.6 years (mean ± SD), mean duration of diabetes 11.0 ± 2.9 years and 10 comparable non-diabetic controls (6 males, 4 females) aged 29.5 ± 4.1 years were studied. The diabetic patients were divided into three groups according to

Table 4.1

Clinical details of diabetic patients and control subjects

Group	N	Age (years)	Duration of diabetes (years)	Number of patients with background retinopathy	Platelet count (x109/l)	Plasma glucose (mmol/l)
Control	10(6M,4F)	29.5±4.1	-	-	295±36	4.5±1.1
Diabetic						
HbA_1<9%	8(5M,3F)	27.4±4.1	9.5±2.6	5	287±47	10.9±3.6
HbA_1= 10-13%	18(9M,9F)	30.5±3.5	11.6±2.9	9	300±54	12.1±4.1
HbA_1>13%	6(4M,2F)	28.6±2.7	11.4±3.0	4	279±30	11.7±2.9

Values expressed as mean ± SD

their prevailing glycaemic control (group A, $HbA_1 \leq 9\%$; group B, HbA_1 = 10-13%; group C, $HbA_1 > 13\%$). None were taking medication other than insulin. All had normal renal function (plasma creatinine <120 μmol/l, urine Albustix negative), were normotensive (BP <140/90) and had easily palpable peripheral pulses. Retinopathy was assessed after mydriasis, and nine of the patients had background retinopathy (ranging from a few microaneurysms to exudative changes) and they were distributed through the three groups (Table 4.1).

4.3 Methods

4.3.1 Platelet density analysis

Working solutions of Percoll (Pharmacia, Piscataway, NJ) were prepared with phosphate-buffered saline (pH 7.4) containing 0.01M EDTA as dilutent. Percoll consists of colloidal silica particles of 15-30nm diameter which have been coated with polyvinylpyrrolodone. Ten millilitres of each solution were used to prepare continuous linear gradients covering the range 1.04- 1.12g/ml. The gradients were calibrated with coloured marker beads (Pharmacia). Blood samples (5ml)

were collected into EDTA (final concentration 0.15%) and prostaglandin (PGE_1) was then added to each sample (final concentration 0.2 μg/ml). The blood was diluted 1:1 (vol/vol) with 0.9% saline before carefully layering 2.5ml onto a Percoll gradient. Gradients were spun for 60 minutes at 2000g (at room temperature) and then fractionated by upward displacement of the gradient with stock Percoll. Platelet counts were performed with a Coulter Plus Z_β (Coulter Electronics Ltd, Luton, UK). The average recovery of platelets from gradients was 76%.

4.3.2 Measurement of intraplatelet nucleotides

Platelet nucleotides were extracted from platelet-rich plasma (PRP) with 1 vol of a solution of trichloracetic acid (10% wt/vol) and EDTA (4mM) for 10min at 4°C. Nucleotides were then assayed by the method of Holmsen et al (1972) with the kit and reagents supplied by LKB (South Croyden, UK).

4.3.3 Measurement of intraplatelet ß-thromboglobulin

ß-thromboglobulin was assayed by radioimmunoassay with the method of Bolton et

al (1976). The amount of ßTG present in platelets was estimated after lysis of EDTA-PRP (with a known platelet count) with Triton X-100.

4.3.4 Glycosylated haemoglobin

The glycosylated haemoglobulin (HbA_1) was carried out by electrophoresis with commercially available agar (Corning Medical, Halstead, UK) as described by Read et al (1980).

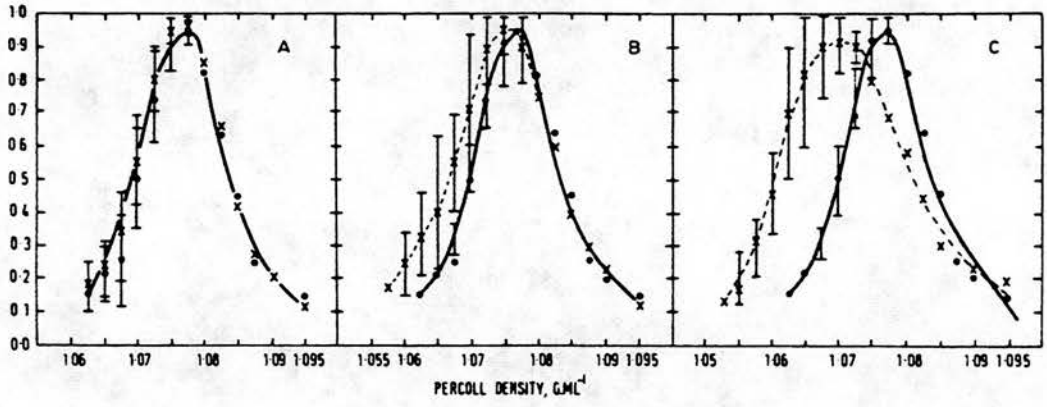
4.3.5 Statistical analysis

Statistical analysis of the density profiles was by Spearman rank-correlation analysis.

4.4 Results

Under the conditions used in the study, normal platelets layered onto Percoll formed a band from 1.0625g/ml to 1.0925g/ml with a mean density of

Figure 4.1



Platelet-density profiles of normal (solid curves) and diabetic (dashed curves) groups. Results expressed as mean ± SD.
A, HbA_1 ≤ 9%; B, HbA_1 = 10-13%; C, HbA_1 > 13%

Table 4.2

Intraplatelet nucleotides and ßTG levels

Groups	N	ATP (nmol/10^8 platelets)	ADP (nmol/10^8 platelets)	ATP/ADP	ßTG (ng/10^8 platelets)
Control	10	6.3±1.6	4.0±1.3	1.7±0.6	54±4
Diabetic					
HbA1< 9%	8	6.6±1.4	3.7±1.0	1.9±0.7	54±14
HbA1= 10-13%	18	7.6±1.8	4.3±1.0	1.9±0.7	56±14
HbA1> 13%	6	6.1±1.3	3.3±1.0	1.9±0.5	55±20

Values are expressed as mean ± SD

1.0775g/ml; the distribution of platelets on gradients was bell shaped. In diabetic patients with an HbA_1 ≤9%, the platelet density profile was superimposable with that of normal subjects. However, in the two diabetic groups with a HbA_1 ≥10%, there was a significant increase in the proportion of low density platelets observed on Percoll ($p< 0.0002$) and the mean platelet density was reduced to 1.0750g/ml ($10 < HbA_1 < 13\%$) and 1.0700g/ml (HbA_1 >13%) (Figure 4.1). Microscopic examination of gradient fractions did not show any evidence that platelet aggregation during centrifugation.

There was no significant difference in the intraplatelet nucleotides (ADP and ATP) and ß-TG contents of the diabetic patients and control subjects (Table 4.2).

4.5 Discussion

When a suspension of particles is centrifuged, the sedimentation rate of the particles is proportional to the force applied. The physical properties of the solution will also affect the sedimentation rate. At a fixed centrifugal force and liquid viscosity, the sedimentation rate is proportional to the size of the particle

and the difference between its density and the density of the surrounding medium.

The equation for the sedimentation of a sphere in a centrifugal field is:

$$v = \frac{d^2 (p_p - p_l) \times g}{18\eta}$$

where v = sedimentation rate

d = diameter of the particle

p_p = particle density

p_l = liquid density

η = viscosity of the medium

g = centrifugal force

From this equation the following relationships can be observed:

(i) sedimentation rate of a particle is proportional to its size

(ii) sedimentation rate is proportional to the difference between the density of the particle and that of the surrounding medium

(iii) sedimentation rate is zero when the density of the particle is equal to the density of the surrounding medium

(iv) sedimentation rate decreases as the viscosity of the medium increases

(v) sedimentation rate increases as the centrifugal force increases

Rate zonal centrifugation and isopycnic centrifugation are the two types of centrifugation used with Percoll. With rate zonal centrifugation, the size difference between particles is the parameter that determines separation. As can be seen from the above equation, large particles move faster through the gradient than small particles, and the density range is chosen so that the density of the particles is greater than the density of the medium at all points during the centrifugation. The run is terminated before the separated zones reach the bottom of the tube (or their equilibrium positions).

In this study, the isopycnic centrifugation technique was used. With this, the density range of the gradient medium encompasses all the densities of the sample particles. Each particle sediments to an equilibrium position in the gradient where the gradient density is equal to the density of the particle (isopycnic position). Thus in this type of separation, the particles are separated solely on the basis of differences in density, irrespective of size.

Previous studies of platelet-density heterogeneity have used various density-

gradient separation media such as Stractan (Mezzano et al, 1981; Boneu et al, 1982), Ludox-PVP (Penington et al, 1976), bovine serum albumin (Charmatz and Karpatkin, 1974) and sucrose (Booyse et al, 1968). Recently, Percoll has been introduced as an alternative medium for the preparation of platelet subpopulations of various densities (Martin et al, 1983), and when used in continuous gradients it appears to overcome the problems associated with viscous density-gradient media and discontinuous gradients. With discontinuous gradients, subpopulations of platelets appear to be trapped at the step interface, and with both this type of gradient and viscous density-gradient media, the time taken to reach equilibrium is substantially increased (Martin and Trowbridge, 1982). In this study, the formation of platelet aggregates during centrifugation was inhibited by the addition of PGE_1 to blood samples and the use of suitably buffered Percoll.

Using density analysis, this study demonstrated an increase in low-density platelets in the peripheral blood of poorly controlled diabetic patients. However, there was no correlation between the presence of increased numbers of low-density platelets and the presence of microangiopathy in the form of retinopathy. Further investigations revealed normal levels of alpha-granules (ßTG) and dense granule (ADP/ATP) contents in the platelets of these individuals. The increase in

the number of low density platelets may occur as a result of the influence on platelet density of platelet aging (Thomson et al, 1983; Blajchman et al, 1981; Watson and Ludlam 1986), the platelet membrane (Rand et al, 1983), or other intraplatelet protein constituents. Particularly, platelet protein constituents may undergo non-enzymatic glycosylation (Brownlee et al, 1984), similar to erythrocyte glycosylation (Agarwal et al, 1985), causing an alteration in platelet density. Alternatively, it has been demonstrated that the proportion of megathrombocytes present in the peripheral blood is increased in diabetes mellitus and in other conditions in which platelet survival is shortened (Garg et al, 1972; Abrahamson, 1968). The heterogeneity of platelet density may occur as a result of platelets of various sizes having comparable granule contents.

Also, some external factor may have contributed to the result in this study. Diabetic platelets have been shown to possess a refractoriness to the inhibitory action of prostaglandins (Betteridge et al, 1982; Davi et al, 1982). PGE_1 is effective in preventing aggregation of normal platelets on gradients during centrifugation. The diabetic platelets, however, may not have been as responsive to PGE_1, and it is possible that a small loss of granule content may have occurred despite the mild centrifugation conditions. If this were the case, it would suggest

that prevailing metabolic control influences the degree of platelet responsiveness to inhibiting prostaglandins.

CHAPTER 5

INCREASED PLATELET THROMBOXANE PRODUCTION AND RECEPTOR SENSITIVITY IN DIABETIC PATIENTS WITH PROLIFERATIVE RETINOPATHY

INCREASED PLATELET THOMBOXANE PRODUCTION AND RECEPTOR SENSITIVITY IN DIABETIC PATIENTS WITH PROLIFERATIVE RETINOPATHY

5.1 Introduction

5.2 Subjects

5.3 Methods

5.3.1 Preparation of platelet suspensions

5.3.2 Platelet aggregation

5.3.3 Platelet release reaction

5.3.4 Intra-platelet 3:5' cyclic monophosphate levels

5.3.5 Radioimmunoassay of thromboxane B2

5.3.6 Glycosylated haemoglobin

5.3.7 Statistical analysis

5.4 Results

5.5 Discussion

5.1 Introduction

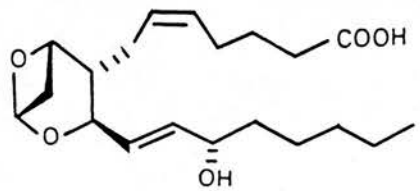
It is recognised that platelets from diabetic patients show an exaggerated response to aggregating agents (Colwell et al, 1983). Hyperaggregation has been reported to be associated with poor glycaemic control (Peterson et al, 1977), increasing age (Lecrubier et al, 1980), and the presence of micro and macrovascular complications (Khosla et al, 1979; Colwell et al, 1977). A number of circulating substances relevant to coagulation, including lipids (Stuart et al,1980; Jones et al, 1986), fibrinogen (Cederholm-Williams et al, 1981) and von Willebrand factor (Lamberton et al, 1984) have also been reported as abnormal in diabetic patients.

Where a possible mechanism for enhanced platelet aggregation has been investigated, the most consistent finding has been the increased production of aggregation-induced thromboxane A_2 (TXA_2) (Ziboh et al, 1979; Halushka et al, 1981; Silberbauer et al, 1981). TXA_2, which is the predominant metabolite of platelet arachidonic acid, is a powerful platelet aggregator and vasoconstrictor (Hamburg et al, 1975; Moncada and Vane, 1978). Most studies have employed arachidonic acid itself or an agent such as collagen, which releases endogenous

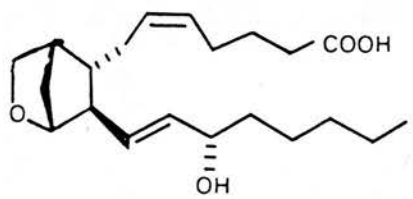
platelet arachidonic acid, to demonstrate enhanced platelet aggregation. Since the production of TXA_2 is known to be increased, it cannot be determined from these studies (Peterson and Gormsen, 1978; Davis et al, 1978) whether improved receptor coupling or an increase in the number of thromboxane receptors may also contribute to this increased sensitivity.

The aims of this study were to confirm platelet hyperaggregability to collagen and to investigate the changes at receptor level in platelets from diabetic patients with established microangiopathy using an analogue of PGH_2, 11,9-epoxymethano PGH_2 (11,9-em PGH_2), which directly stimulates thromboxane receptors (Di Minno et al, 1981; Di Minno et al, 1985), plus a competitive thromboxane receptor antagonist (EP 092) (Jones et al, 1982) (Figure 5.1). Young insulin dependent diabetic patients who had background and proliferative retinopathy, as well as a comparable non-diabetic control group were selected in order to investigate the possible relationship between platelet function and susceptibility to proliferative retinopathy.

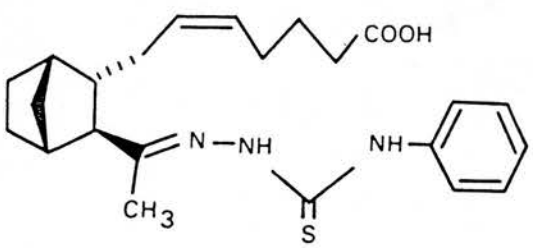
Figure 5.1



THROMBOXANE A_2



11,9—epoxymethanoPGH_2



EP 092

Structures of thromboxane A_2, 11,9-epoxymethano-prostagladin H_2 (thromboxane mimetic) and EP 092 (thromboxane receptor antagonist)

5.2 Subjects

Twenty-four insulin dependent diabetic patients and twelve non-diabetic healthy volunteers were studied. Classification of retinopathy was based on both ophthalmoscopic and fluorescein angiographic appearances. Twelve patients had background retinopathy and twelve had proliferative retinopathy. In the background retinopathy group, three patients had scattered microaneurysms and haemorrhages, seven patients had hard exudative changes and two patients had ischaemic preproliferative changes. The three groups were comparable for age and sex, and the diabetic groups were matched for duration of diabetes (Table 5.1). None of the diabetic subjects were taking medication other than insulin, all were clinically free of nephropathy (plasma creatinine <120 μmol/l, urine Albustix negative), were normotensive (BP<140/90) and all had easily palpable peripheral pulses.

Table 5.1

Age, duration of diabetes, plasma glucose, HbA_1 and platelet counts in controls and diabetic retinopathy groups

Group	Number of subject	Age (years)	Duration of diabetes (years)	Plasma glucose (mmol/l)	HbA1 (%)	Platelet Count ($x10^9/l$)
Control	12(6M,6F)	26.7(1.3)	-	4.6(0.3)	7.6(0.3)	274(11)
Background Retinopathy	12(6M,6F)	27.6(0.9)	15.2(1.1)	10.5(1.0)	11.8(0.7)	257(16)
Proliferative Retinopathy	12(6M,6F)	27.0(1.5)*	14.6(1.4)**	11.6(1.7)**	12.6(0.7)**	245(1.2)**

Results expressed as mean (SEM)

* NS between the three groups
** NS between the diabetic groups

5.3 Methods

5.3.1 Preparation of platelet suspensions

All manipulations were carried out at room temperature. Blood (100mls) was collected between 8.00 and 9.00 hours from fasted diabetic patients and control subjects into plastic centrifuge tubes containing acid-citrate dextrose (1ml for 5ml blood) and centrifuged at 120g for 20 minutes. The platelet-rich plasma (PRP) was then pre-incubated with [14^{o}C] 5-hydroxytryptamine (final concentration 1 μg/ml) for 30 minutes. The platelet suspensions were kept under an atmosphere of 95% 0_2/5% CO_2 which kept the pH constant, and therefore prevented the fall in sensitivity to thromboxane-like action which occurs with time (Kerry and Paton, 1984).

5.3.2 Platelet aggregation measurements

Platelet aggregation was measured by the turbidometric method of Born (1962), using a Bryson Aggregometer (II Upchurch and Co Ltd). The changes in light transmission of the platelet suspension were recorded on a potentiometric recorder (Servoscribe Is) The cell block was maintained at 37^{o}C and stirring was achieved with a stainless steel rod.

Each cuvette contained:

	Vol (ml)
Platelet-rich plasma	0.5
Krebs solution	0.3
Saline (0.9%)	0.1 - 0.2
Vehicle	0.1 - 0.2
Total volume	1ml

Dose response curves for aggregation induced by collagen and 11,9-em PGH were determined both in the presence and absence of the cyclooxygenase inhibitor, Froben (10 μmol/l). This eliminated the endogenous production of TXA_2 and allowed assessment of aggregation in the absence of the secondary aggregation wave. In addition, the shift in the dose-response curve for aggregation using a fixed concentration of the competitive TXA_2 receptor antagonist EP 092 (0.25 μmol/l) was established. Each aliquot was warmed to 37°C for 2 minutes with constant stirring (1100 rev/min) prior to the addition of the aggregating agents. If used, Froben or EP 092 were added at time zero, 2 minutes before the addition of the aggregating agent.

5.3.3 Measurement of 5-hydroxytryptamine (5-HT) release from platelets

Platelet rich plasma was pre-incubated with radiolabelled 5HT(5-hydroxy-

(side-chain) 2-[14C]-tryptamine) at 37°C for 30 minutes. The [14C]-5HT is actively taken up by the platelet into the dense granular bodies during this pre-incubation period. Following the incubation period, platelet rich plasma was used in aggregation studies as described. In order to estimate [14C]-5HT release, at the end of the four minute period, 200 µl samples in duplicate were withdrawn from the cuvette and added to 0.8ml ice cold EDTA (0.4% w/v in 0.9% saline) in an Eppendorf tube. The quenched samples were centrifuged immediately at 16000g (20°C for 30 seconds) in a bench Eppendorf centrifuge. Subsamples (0.5ml) of the cell free supernatant were then transferred into 10ml of scintillant (PCS-Amersham-Searle/toluene 2:1) and conventional scintillation counting carried out. The [14C]-5HT released was expressed as a percentage of the amount of [14C]-5HT taken up by the platelets.

5.3.4 Measurement of intraplatelet 3:5 cyclic monophosphate (cAMP)

This assay is based upon competition between unlabelled cAMP and a fixed quantity of ^{3}H labelled cAMP for binding to a protein which has high specificity and affinity for cAMP. PGE_1 (0.2 µmol/l) was incubated with the platelet suspension (1ml) for 37°C for 30 seconds. The reaction was quenched by addition of 2ml ethanol and

5 mins later the sample was centrifuged at 2000g. The supernatant was removed and the pellet suspended in 1ml ethanol:water (2:1) and centrifugation repeated. The combined supernatants were evaporated to dryness at 55^{o}C under a stream of nitrogen. The residue was dissolved in 0.5ml assay buffer and centrifuged at 12,000g for 30 seconds to remove insoluble material. Duplicate 50 µl samples were removed and their cAMP content measured by a protein- binding assay (Gilman 1970, Armstrong et al, 1985). The extraction procedure gave a mean recovery of 87 ± 1.4% (16 replicates); the inter-assay coefficient of variation was 11% while the intra-assay coefficient of variation was 4%.

5.3.5 Radioimmunoassay of thromboxane B_2

The underlying principle of radioimmunoassay depends upon competitive binding, where labelled and unlabelled antigen compete for antibody binding sites. The distribution of radionucleotide counts in the bound and free states is proportional to the relative concentration of unlabelled antigen. TXB_2 concentrations are thought to reflect the levels of the active precursor TXA_2 present from biosynthetic transformations of arachidonic acid.

The TXB_2 antibody used was obtained commercially from The Pasteur Institute, France, in freeze dried form, and before use was reconstituted in phosphate buffer pH 7.5. TXB_2 produced in response to exogenous collagen (2 µg/ml) was measured in duplicate using a standard double antibody technique. Collagen was added to 1ml PRP and incubated at 37°C for 30 seconds. The reaction was then quenched with 2M HCl and the TXB_2 then extracted into ethylacetate (Et Ac). The Et Ac was then removed after centrifugation at 2000g for 5 minutes and stored at -20°C until assayed. The intraassay coefficient of variation was 4.6% while the interassay coefficient of variation was 3.2%.

5.3.6 Measurement of glycosylated haemoglobin (HbA_1)

Glycosylated haemoglobin was estimated using commercially available agar plates (Corning Medical, Halstead, UK) as described by Read et al (1980), the normal range being 6 - 8%.

5.3.7 Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis of the dose ratios levels

was by Kruskal-Wallis one-way analysis of variance by ranks. Comparison between the groups for the cAMP and TXB_2 results were by Students' t-test with the Bonferonni correction.

5.4 Results

There was no difference in the concentration of collagen EC_{50}, required to give 50% of the maximum aggregation wave between the two diabetic groups and the control group (Table 5.2). Similarly, the thromboxane receptor antagonist EP 092 shifted all three curves to a similar extent, as shown by the new EC_{50} values in the presence of EP 092, and by the dose ratios (DR = EC_{50} in the presence of EP 092/EC_{50} collagen alone). The cyclooxygenase inhibitor Froben produced a more marked shift of the control aggregation curves than observed with EP 092. Froben appeared to be slightly less effective in the proliferative retinopathy group, but this was not statistically significant.

There was, however, a significant difference between the three groups in the concentration of 11,9-em PGH_2 required to induce 50% aggregation response. Less 11,9-em PGH_2 was required for the proliferative retinopathy group

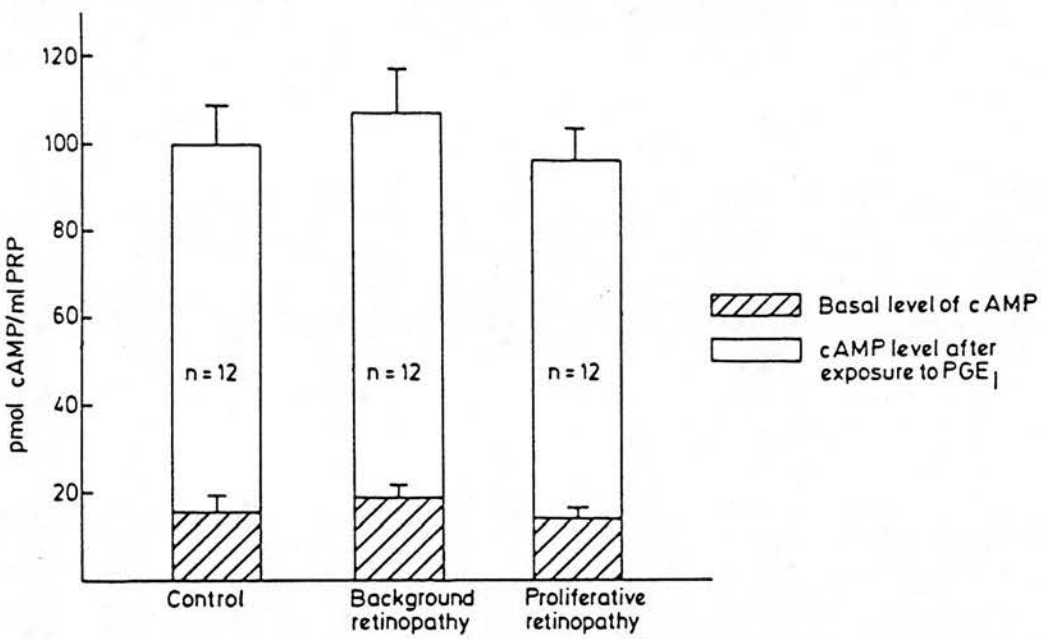
compared with either the control or retinopathy groups ($P < O.005$). Furthermore, EP 092 produced a significantly larger shift in the aggregation curve in the proliferative retinopathy group as judged by the dose ratio when compared with the other two groups ($P < O.005$). In the presence of Froben no difference was found in the three groups (Table 5.3).

5-Hydroxytryptamine release in response to both collagen and 11,9-em PGH_2 showed no difference between the three groups. (Table 5.4).

There was no difference in the basal level of cAMP found in platelets from the three groups or in the rise in cAMP induced by PGE_1 (Figure 5.2).

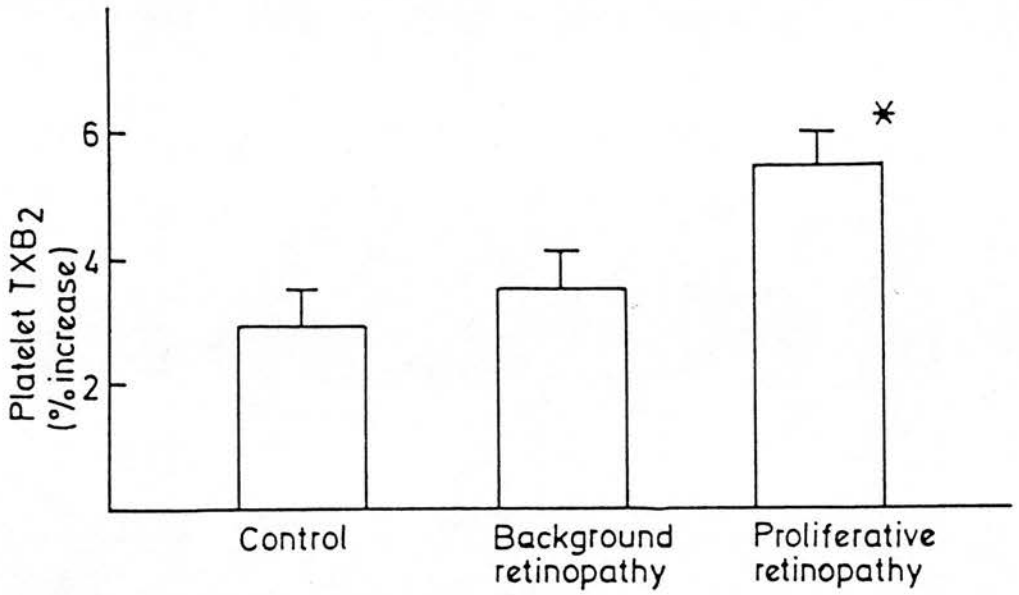
There was a significant increase in the platelet TXB_2 production following exposure to collagen in the proliferative retinopathy ($P< 0.05$) compared with the background retinopathy and control groups (Figure 5.3).

Figure 5.2



Basal platelet cAMP levels and cAMP levels after exposure to prostaglandin E_1 (PGE_1) in control and diabetic retinopathy groups. Results given as mean $\pm$ SEM.

Figure 5.3



Percentage increase in platelet thromboxane B_2 production after exposure to collagen in the control and diabetic retinopathy groups. Results expressed as mean $\pm$ SEM. (* $p < 0.05$).

Table 5.2

Results of aggregation EC_{50} and dose ratio for collagen in the presence of EP 092 (0.25 μmol/l) and Froben (10 μmol/l).

Group	Collagen EC_{50} (μg/ml)	Collagen +EP092 EC_{50} (μg/ml)	Dose Ratio	Collagen +Froben EC_{50} (μg/ml)	Dose Ratio
Control	0.8(0.1)	1.9(0.3)	2.4(0.3)	8.3(1.9)	9.8(2.0)
Background Retinopathy	0.74(0.1)	1.8(0.3)	2.7(0.5)	6.6(1.6)	10.8(3.4)
Proliferative Retinopathy	1.1(0.2)	1.9(0.4)	1.9(0.2)	4.9(0.7)	5.0(0.5)
	NS	NS	NS	NS	NS

Results expressed as mean (SEM).

Table 5.3

Results of aggregation EC_{50} and dose ratio for 11,9em-PGH2 in the presence of EP 092 (0.25 µmol/l) and Froben (10 µmol/l).

Group	11,9emPGH2 EC_{50} (µg/ml)	11,9emPGH$_2$ +EP 092 EC_{50} (ug/ml)	Dose Ratio	11,9emPGH$_2$ + Froben EC_{50} (µg/ml)	Dose Ratio
Control	99.4(10.0)	115.3(5.2)	1.7(0.1)	124.7(9.4)	1.3(0.1)
Background Retinopathy	85.4(11.5)	193.8(28.4)	2.6(0.6)	120(12.9)	1.5(0.4)
Proliferative Retinopathy	63.5(6.6) *	176.0(20.2)	3.2(0.6) *	100.4(8.0)	1.6(0.1)

Results expressed as mean (SEM)

* $p < 0.005$, significant difference between proliferative retinopathy and background retinopathy group

Table 5.4

Results of platelet release in control and diabetic retinopathy groups due to collagen (2 μg/ml) and 11,9 em-PGH2 (250 μg/ml) alone, and in the presence of EP 092 (0.25 μmol/l) and Froben (10 μmol/l)

Group	Collagen	Collagen + EP092	Collagen + Froben	11,9emPGH$_2$	11,9emPGH$_2$ + EP092	11,9emPGH$_2$ + Froben
Control	58.8(2.2)	41.1(3.6)	19.8(1.1)	34.9(1.0)	30.9(1.4)	32.7(1.3)
Background Retinopathy	51.8(4.5)	39.3(3.9)	21.6(1.4)	30.8(2.9)	26.7(2.9)	29.3(2.3)
Proliferative Retinopathy	55.3(5.7)	30.7(4.1)	18.4(2.4)	32.8(2.9)	28.7(3.4)	30.1(1.7)

Results expressed as % total [^{14}C]-5HT released/[^{14}C]-5HT taken up by platelets at pre-incubation and given as mean (SEM).

5.5 Discussion

This study demonstrated that platelets from insulin-dependent diabetic patients with proliferative retinopathy are more sensitive to 11,9-em PGH_2 than platelets from insulin-dependent diabetic patients with background retinopathy and control subjects. However unlike previous studies in diabetic patients (Sagel et al, 1975; Silberbauer et al, 1981; Janka and Demmel, 1981; Jones et al, 1985), increased platelet sensitivity to collagen was not found, whether sensitivity was measured as platelet aggregation or 5-hydroxytryptamine release. In non-diabetic subjects endogenous PGH_2 and TXA_2 production are the major components of collagen-induced aggregation (Vergaftig et al, 1981). The aggregation EC_{50} for collagen was similar to the two diabetic and control groups, with the reversible thromboxane antagonist EP 092 producing a similar shift of the aggregation wave in each group. The cyclooxygenase inhibitor Froben appeared to be slightly less effective in the proliferative group than in the background retinopathy and control groups, but this did not achieve statistical significance. The slight decrease in the efficiency of Froben in this group is consistent with increased thromboxane production in response to collagen without alteration in the aggregation EC_{50} level. From these observations it would appear that the mechanism for collagen induced

aggregation is similar in both diabetic patients and control subjects.

Age is an important determinant of platelet function in both non-diabetic and diabetic patients (Lecrubier et al, 1980). During recruitment to this study particular attention was paid to careful age restriction and comparability. This may account in part for the apparent discrepancy in platelet collagen sensitivity in diabetic patients in this study compared with other studies (Sagel et al, 1975; Silberbauer et al, 1981; Janka and Demmel, 1981; Jones et al, 1985).

In contrast, platelets from the diabetic group with proliferative retinopathy both produce more thromboxane A_2 and were more responsive to the thromboxane mimetic 11, 9-em PGH_2. The increase in sensitivity was reduced in the presence of the cyclooxygenese inhibitor, Froben. In non-diabetic subjects the major mechanism of the action of the mimetic does not involve activation of the thromboxane generating system (Hamburg et al, 1975; Moncada and Vane, 1978). It would appear however, that in diabetic patients with proliferative retinopathy, that the increased sensitivity is not a direct effect of the 11, 9-em PGH_2 on the receptor. The actual mechanism remains undefined, but is probably not due to the increased endogenous thromboxane produced during secondary aggregation.

There was no difference between either the basal level of platelet cAMP levels or increased cAMP level after exposure to PGE_1 between the diabetic patients and control subjects. This is in agreement with the study by Shepherd et al (1983), investigating PGI_2 binding to platelets but in sharp contrast to that of Lagarde et al (1981), where lower basal levels of cAMP and a diminished response to inhibiting prostaglandins were reported. The likely explanation is that in our study and that reported by Shepherd et al (1983) the subjects were young and age-matched, whereas in the report by Lagarde et al (1981) the ranges of age, duration of diabetes and vascular complications were very wide.

In conclusion, this study demonstrates that platelets from insulin-dependent diabetic patients with proliferative retinopathy both have increased thromboxane production during aggregation and increased sensitivity to a thromboxane mimetic; these may be factors in the development of proliferative retinopathy.

CHAPTER 6

CHANGES IN SOME ASPECTS OF PLATELET FUNCTION WITH IMPROVEMENT OF GLYCAEMIC CONTROL IN INSULIN-DEPENDENT DIABETIC PATIENTS

CHANGES IN SOME ASPECTS OF PLATELET FUNCTION WITH IMPROVEMENT OF GLYCAEMIC CONTROL IN INSULIN-DEPENDENT DIABETIC PATIENTS

6.1 Introduction

6.2 Subjects

6.3 Methods

6.3.1 Preparation of platelet suspensions

6.3.2 Platelet aggregation

6.3.3 Radioimmunoassay of thromboxane $ß_2$

6.3.4 Measurement of intraplatelet 3 : 5 cyclic monophosphate

6.3.5 Measurement of plasma ß-thromoglobulin

6.3.6 Glycosylated haemoglobin

6.3.7 Statistical analysis

6.4 Results

6.5 Discussion

6.1 Introduction

Numerous studies have suggested that platelets from diabetic patients are hypersensitive to a variety of pro- aggregating agents (Jones & Peterson, 1981; Colwell et al,1983). As discussed in the previous chapter, the abnormal platelet aggregatory response is often associated with an increase in platelet TXA_2 synthesis (Buktus et al, 1980; Halushka et al, 1981). The concept of increased platelet TXA_2 production in diabetes is also consistent with the finding that the platelet aggregation response to arachidonic acid in diabetic patients, is less well inhibited by the TXA_2 antagonist, 13-azaprostanic acid (Le Breton et al, 1979; Mayfield et al, 1985).

Prostacyclin (PGI_2), which is produced in the vascular endothelium has both potent platelet anti-aggregating and vasodilator activity (Moncada and Vane, 1978; Gryglewski, 1980). However, in vitro studies have suggested that there is a reduction in endothelial PGI_2 production in diabetic patients (Johnson et al, 1979; Aanderud et al, 1985), although in vivo measurements contradict this finding (Mourits Anderson et al, 1986). In addition, it is reported that platelets from diabetic subjects have reduced sensitivity to PGI_2 (Schernthaner et al, 1981;

Betteridge et al, 1982; Davi et al, 1982) further promoting hyperaggregability.

There is some evidence that improved glycaemic control may reverse some aspects of the hyperaggregability of diabetic platelets (McDonald et al, 1982; Giugliano et al, 1982; Evans et al, 1982). Therefore, the aims of this study were to investigate the changes that occur in ADP-induced platelet aggregation, platelet sensitivity to a stable PGI_2 analogue (Iloprost), aggregation induced platelet TXB_2 production, platelet cAMP levels and plasma ßTG levels in nine young insulin-dependent diabetic patients, in which the glycaemic control was significantly improved in one group (n=5) over a six month period.

6.2 Subjects

Nine moderately well controlled young male insulin- dependent diabetic patients participated in the study. Five patients ("tight" control group) improved their glycaemic control with a fall of HbA_1, from 11.9 ± 1.0% to 9.0 ± 0.8% while the HbA_1 in four patients ("usual" control group) did not change significantly. Ophthalmoscopy following mydriasis revealed that three patients in the "tight"

Table 6.1

Cinical details of "tight" and "usual" control patients

Group	Age (yrs)	Duration of diabetes (yrs)	HbA_1 (%) To	T_1
"Tight" control (n=5)	30.8 (28.6-32.8)	8.8 (6.2-10.6)	11.9 (10.1-12.8)	9.0*
"Usual" control (n=4)	29.8 (27.9-32.1)	8.6 (6.3-9.5)	11.4 (9.6-12.2)	11.9 (9.4-13.0)

Results are expressed as mean (range)

* $p < 0.05$

control group and two patients in the "usual" control group had mild background retinopathy. All the patients were normotensive (BP <140/90), had clinically normal renal function (plasma creatinine <120 μmol/l, urine Albustix negative) and had easily palpable peripheral pulses. None were taking medication other than insulin. There was no significant difference in the mean plasma glucose or the mean platelet count at the time of the venesection in either group.

6.3 Methods

6.3.1 Preparation of platelet suspensions

Platelet suspensions were prepared as described in the previous chapter.

6.3.2 Platelet aggregation methods

Platelet aggregation was measured by the method of Born (1962), using a Bryson Aggregometer (II Upchurch and Co Ltd). Each aliquot of PRP (0.5 mls) and Krebs Solution (0.3 mls) was warmed to 37^{o}C for 2 minutes prior to the addition of the

aggregating agent, ADP. The stable prostacyclin analogue (Iloprost, 1.5 x 10^{-9}M) was added at time 0, 2 minutes prior to the addition of ADP. Dose response curves were constructed for aggregation induced by ADP alone, and in the presence of Iloprost.

6.3.3 Radioimmunoassay of thromboxane B_2

Collagen (2 µg/ml) induced platelet thromboxane production was measured in duplicate by radioimmunoassay using a standard double antibody technique.

6.3.4 Measurement of intraplatelet 3:5 cyclic monophosphate

Basal platelet cAMP and the rise in cAMP induced by Iloprost (1.0 x 10^{-9}M) were measured by a protein binding assay as described in the previous chapter.

6.3.5 Measurement of plasma ß thromboglobulin levels

Plasma ß thromboglobulin levels were measured using radioimmunoassay.

6.3.6 Glycosylated haemoglobin

Glycosylated haemoglobin was estimated using commercially available agar plates, the normal range being 6-8%.

6.3.7 Statistical analysis

Results are expressed as mean (range) and statistical analysis was performed using the Wilcoxon rank sum test.

6.4 Results

With improvement of glycaemic control there was no significant change in the concentration of ADP required to produce 50 percent of the maximum aggregation wave response (EC_{50}). However, with improved glycaemic control, there was a significant increase in the responsiveness of the platelets to Iloprost ($p < 0.05$). There was no difference in the platelet TXB_2 production between the two groups at the start of the study (To) ($p<0.05$) (Table 6.1). There was, however, an increase in collagen-induced platelet thromboxane production with improved

Table 6.2

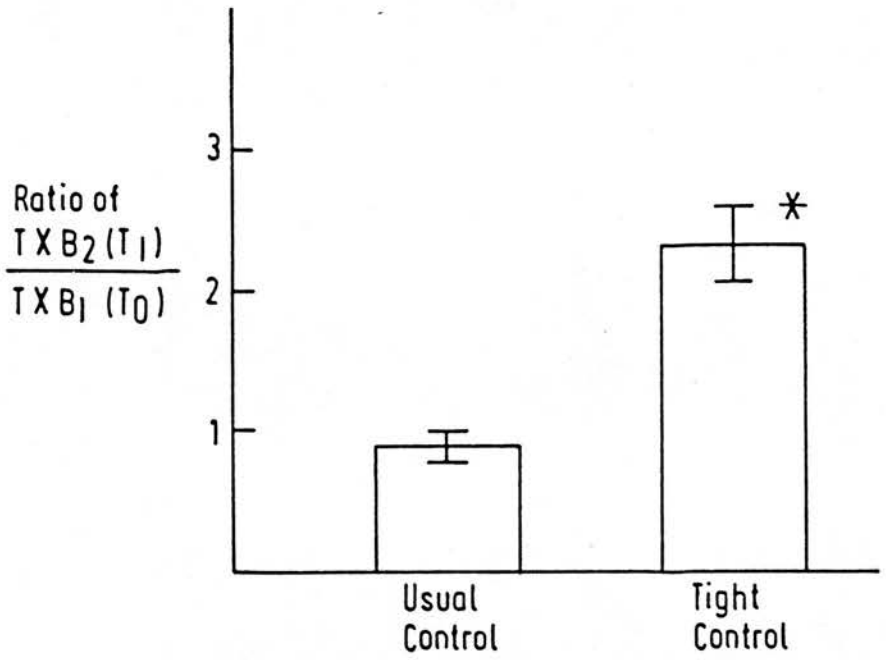
Platelet aggregation EC_{50} for ADP, dose ratio for inhibition by Iloprost (1.5×10^{-9} M) and platelet cAMP level (pmol cAMP/ml PRP) after exposure to Iloprost at To and T1.

Groups	EC_{50} for ADP (10^{-7} M)		ADP Dose-ratio for inhibition by Iloprost		Platelet cAMP level after exposure to Iloprost	
	To	Ti	To	Ti	To	Ti
"Tight" control (^=5)	8.0 (5.4-0.4)	6.0 (3.2-8.3)	1.3 (1.1-1.9)	3.3* (2.5-4.2)	1.2 (1.0-1.4)	1.2 (0.9-1.6)
"Usual control (^=4)	6.0 (4.1-8.6)	6.5 (4.2-8.8)	2.5 (1.6-2.8)	1.9 (1.4-2.7)	1.3 (1.0-1.7)	1.2 (1.0-1.5)

Results expressed as mean (range)

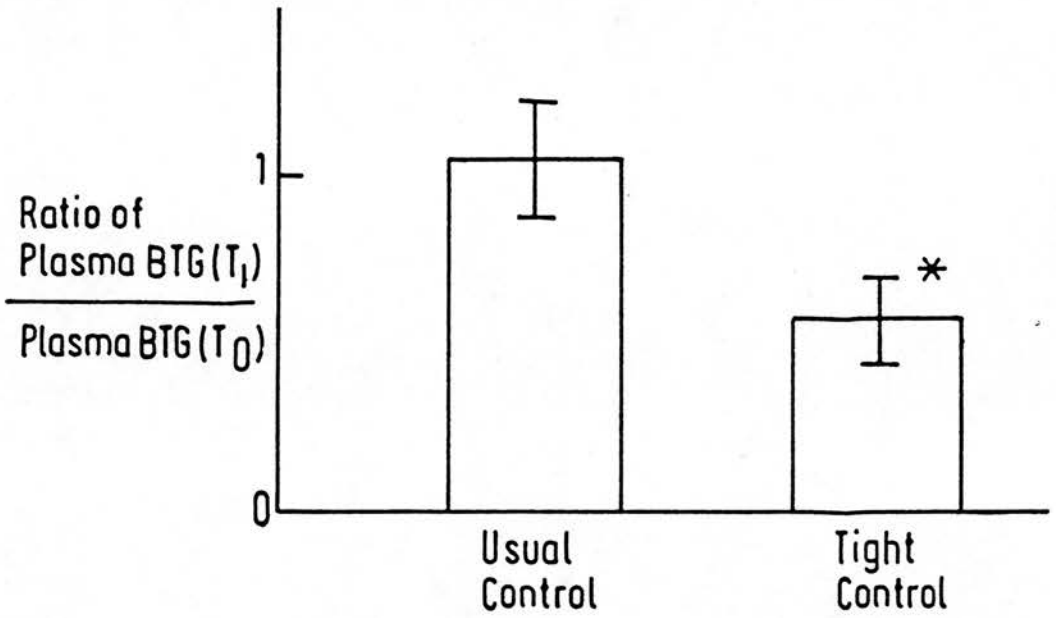
* $p<0.05$

Figure 6.1



Ratio of platelet thromboxane (TX)B_2 production of 6 months (T_1) with platelet TXB_2 production at start of study (To) in the usual control and tight control groups. Results expressed as mean $\pm$ SEM. (* $p < 0.05$).

Figure 6.2



Ratio of plasma B-thromboglobulin (BTG) level at 6 months (T_1) with plasma BTG level at start of study (To) in the usual and tight control groups. Results expressed as mean $\pm$ SEM. (* $p < 0.05$).

glycaemic control ($p < 0.05$) (Figure 6.1). There was no significant difference between the platelet cAMP levels before or after exposure to Iloprost (Table 6.2), but, there was a significant reduction in the plasma ßTG levels after six months of improved glycaemic control ($p < 0.05$).

6.5 Discussion

In the present study, using a multiple injection regime, five out of nine patients attained improved glycaemic control. These patients underwent thorough dietary education, and undertook frequent home blood glucose monitoring. However, despite the significant improvement in the glycosylated haemoglobin over the six months, normoglycaemia was not obtained. Therefore, the changes that occurred in platelet function in this study may be different to those that would have occurred had normoglycaemia been achieved.

It has been recognised that some aspects of the hyperaggregation of diabetic platelets may be reversed following insulin treatment (McDonald et al, 1982; Guigliano et al, 1982; Evans et al, 1982; Hiramatsu et al, 1987). Numerous

mechanisms have been put forward to account for these changes, including, reduction in plasma glucose per se (Jones et al, 1986), alteration in triglyceride and free fatty acids levels due to enhancement of lipoprotein lipase activity (Sadur and Eckel, 1982), the direct effect of hyperinsulinaemia on platelet metabolism (Hiramatsu et al, 1986), and also possibly through platelet phospholipid changes (Jones et al, 1983).

In this study, the platelets became more sensitive to the prostacyclin mimetic, Iloprost. Janka and Demmel (1981), who studied a heterogeneous group of non-insulin diabetic patients demonstrated that with improvement of glycaemic control there was an increase in prostacyclin sensitivity mediated through raised cAMP levels. However, this study showed that with improved glycaemic control there was no significant difference between the Iloprost induced rises in platelet cAMP. Therefore it is probable that the increase in Iloprost sensitivity occurred at a later stage than the prostacyclin receptor adenyl-cyclase complex, and is perhaps due to more efficient sequestration of internal free calcium.

This study has also shown that with improved glycaemic control there was a fall in plasma ßTG levels indicating that over this period there was a reduction in platelet

activation. However, the platelets produced more TXB_2 during aggregation and in keeping with this there was a slight, but non-significant increase in the aggregation EC_{50} for ADP, Reduced prostaglandin production from endogenous platelet arachidonic acid (Janka and Demmel, 1981), along with reduced thromboxane synthesis have been reported in diabetic patients (Tindall et al, 1981; Ylikorka et al, 1981). Jones et al, (1983) demonstrated reduced arachidonic levels in the platelet membrane phospholipids of diabetic patients, and this correlated negatively with the glycosylated haemoglobin. Therefore, the increase in collagen-induced TXB_2 production associated with improved glycaemic control may be due to increased availability of arachidonic acid from membrane phospholipids (Jackson et al, 1984).

It is well documented that there may be a deterioration in pre-existing microangiopathy in the form of retinopathy after the institution of good diabetic control (Steno Study Group, 1983; Kroc Collaborative Study Group, 1984; Dandona et al, 1985; Dahl-Jorgensen et al, 1985), and the reason remains unclear. Rapid improvement of glycaemia to normal may have a number of pathogenic consequences. Firstly, it may reduce the retinal hyperperfusion which is a characteristic of poorly controlled diabetes (Parving et al, 1983). Secondly,

it significantly reduces the local concentration of glucose which is a major substrate for retinal metabolism. In areas of retinal capillary closure just maintained by diffusion from hyperperfusion and nutrient excess, the combination of reduced perfusion and nutrient concentration, possibly induces retinal deterioration (Keen, 1984). In addition, changes in platelets, for example increased thromboxane production and increase in aggregability (Dandona et al, 1985), may also contribute. Indeed, some of the changes seen in this study are consistent with platelet hyperaggregability contributing to the deterioration in retinopathy associated with improved glycaemic control. Furthermore, the frequency of hypoglycaemia is increased in insulin-dependent diabetic patients undergoing intensive improvement in glycaemic control (Steno Study Group, 1983). As described in chapter 9, hypoglycaemia itself can induce haemostatic and haemodynamic changes which may contribute to further deterioration in microvascular disease (Frier and Hilsted, 1985; Frier, 1986).

Alternatively, the changes described may simply reflect the maintenance of a haemostatic balance. For example, platelets from mature rats have been demonstrated to be more sensitive to prostacyclin as well as having increased aggregability to collagen (Giana et al, 1985). It has been suggested that during ,

compensatory mechanisms operate to counteract increased platelet reactivity (Giana et al, 1985). It is possible that in diabetic subjects undergoing improvement of glycaemic control, a similar process occurs.

CHAPTER 7

EFFECT OF DIET AND ORAL HYPOGLYCAEMIC THERAPY ON PLATELET PARAMETERS IN NEWLY DIAGNOSED NON-INSULIN DEPENDENT DIABETIC PATIENTS

EFFECT OF ORAL HYPOGLYCAEMIC THERAPY ON PLATELET PARAMETERS IN NEWLY DIAGNOSED NON-INSULIN-DEPENDENT DIABETIC PATIENTS

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7.4.1 Glycaemic control

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7.1 Introduction

Despite the heterogeneity in aetiology, the increased predilection for vascular complications is not restricted to any one type of diabetes. Epidemiological evidence in non-insulin-dependent diabetic patients suggests that fasting plasma glucose levels above 6mmol/l are associated with a greater cardiovascular risk (Eschwege et al, 1980), and that once levels are above 8mmol/l there is an increased risk of developing diabetic retinopathy (Jarrett and Keen, 1976). Furthermore, clinically important vascular lesions may be the presenting feature in individuals who have relatively "mild" diabetes (Pirart, 1978; Kannel and McGee, 1979) indicating that there may be a significant delay between the onset of diabetes and diagnosis.

In this study the changes in platelet density profiles, intraplatelet ß-thromboglobulin (ßTG), plasma ßTG levels, intraplatelet cyclic AMP (cAMP) levels, platelet release reaction, platelet thromboxane (TX)B_2 production and plasma fibrinogen levels have been investigated in 24 non-insulin-dependent diabetic patients. These parameters were measured at diagnosis, after a 3-6 week dietary run-in period, and again after 6 months on treatment with either

metformin or gliclazide therapy.

7.2 Subjects

Twenty-four newly diagnosed, non-obese, non-insulin- dependent (age 54.3 ± 6.2 years; mean ± SD) diabetic patients and 12 comparably aged (52.6 ± 7.4 years) controls were studied. The patients had a body weight within the range 90 - 114% ideal (Metropolitan Life Insurance Tables), had normal renal function (plasma creatinine <130 μmol/l; urine Albustix negative), had easily palpable peripheral pulses and were non-smokers. None of the patients had evidence of retinopathy. None were taking medication at entry into the study, particularly significant aspirin ingestion was excluded by plasma aspirin levels at the entry into and completion of the study. All patients had HbA_1 >9% at the end of the dietary run-in period and were therefore randomized into either metformin (Glucophage) or gliclazide (Diamicron) treatment groups, the drugs being given in an open manner (Tables 1 and 2). For the study periods, patients were advised to take a diet low in refined carbohydrate content, moderate in fibre and low in saturated fat. The carbohydrate portion made up approximately 50% of the total energy content. The patients were studied at diagnosis (Week -3/-6), 3 to 6

Table 7.1

Clinical details of the metformin-treated group at diagnosis (week -3/-6), at the end of the dietary run-in period (week 0) and after 6 months on metformin therapy (week 24)

	Week -3/-6	Week 0	Week 24
Age (yrs)	53.1 (5.1)	-	-
Number of patients	12 (6M, 6F)	-	-
Fasting plasma glucose (mmol/l)	15.0 (3.1)	11.8 (3.1)	7.5 (1.7)
HbA_1 (%)	14.3 (2.5)	12.1 (2.4)	7.4 (0.8)
BMI (kg/m^2)	25.0 (1.4)	24.3 (1.4)	24.5 (1.6)
Plasma Cholesterol (mmol/l)	6.9 (1.1)	6.5 (0.9)	5.8 (0.7)
Plasma Triglyceride (mmol/l)	2.2 (0.9)	1.6 (0.5)	1.3 (0.4)

Values expressed as mean (SD)

Table 7.2

Clinical details of the gliclazide treated group at diagnosis (week -3/-6), at the end of the dietary run-in period (week 0) and after 6 months on oral hypoglycaemic therapy (week 24)

	Week -3/-6	Week 0	Week 24
Age (yrs)	55.5 (5.1)	-	-
Number of patients	12 (6M, 6F)	-	-
Fasting glucose (mmol/l)	14.2 (2.6)	12.2 (2.4)	6.4 (1.5)
HbA_1 (%)	13.3 (1.4)	11.7 (1.5)	7.0 (0.8)
BMI (kg/m^2)	23.7 (1.4)	23.1 (1.3)	23.6 (1.4)
Plasma Cholesterol (mmol/l)	7.5 (0.9)	7.0 (0.7)	6.3 (0.8)
Plasma Triglycerides (mmol/l)	2.3 (0.7)	1.9 (0.6)	1.6 (0.5)

Values expressed as mean (SD)

weeks later at the end of dietary run-in period (Week 0) and again after 6 months of oral hypoglycaemic therapy (Week 24).

7.3 Methods

7.3.1 Clinical Assessment

Fasting plasma glucose was measured using a Yellow Springs Glucose Oxidase Analyser. Glycaemic control, assessed by glycosylated haemoglobin (HbA_1), was measured using commercially available agar plates (Read et al, 1980) the normal range between 6-8%. Fasting triglycerides and cholesterol levels were estimated using standard laboratory techniques (Tables 1 and 2).

7.3.2 Platelet Density

Platelet density was measured by the same method described in chapter 4. Platelet counts were performed using a Coulter counter.

7.3.3 Platelet granule content and plasma ßTG levels

As a measure of electron-dense granules, platelet nucleotides (ATP/ADP) were extracted from platelet-rich plasma with 1 vol of a solution of trichloroacetic acid (10% wt/vol) and EDTA for 10 min at 4°C. Nucleotides were then assayed by the method of Holmsen et al (1972) with the kit and reagents supplied by LKB (South Croydon, UK). ß-thromboglobulin (ßTG) was assayed by radioimmunoassay using the method of Bolton et al (1976). The amount of ßTG present in platelets, which reflects platelet alpha-granule content was estimated after lysis of EDTA:PRP (with known platelet count) with Triton X-100.

7.3.4 Platelet TXB_2 production, release reaction, cAMP levels and plasma fibrinogen

Platelet TXB_2 production response to exogenous collagen (2 µg/ml) was measured in duplicate using a standard double antibody technique as described in chapter 5 (Armstrong et al, 1985). Release of platelet dense granule constituents (5HT, ADP) were measured using the radio-labelled isotope prelabelling technique (Armstrong et al, 1985) and basal platelet cAMP and the rise in cAMP by PGE_1

(0.2 μmol/l) were measured by a protein binding assay (Gilman, 1970; Armstrong et al, 1985): the methods are all described in chapter 5. Fibrinogen was measured using the Claus technique (1957)

7.3.5 Statistical analysis

Results are expressed as mean (SD) and the groups were compared using Students' t-tests.

7.4 Results

Tables 7.1 and 7.2 summarise the biochemical profiles (fasting plasma glucose, HbA_1 and fasting plasma triglyceride and cholesterol) and body mass index at diagnosis, after the dietary run-in period and after 6 months in the metformin- and gliclazide- therapy groups. The dosages of metformin and gliclazide ranged from 1.5 - 3.0 (median 2.0) g/day and 80 - 240 (median 160) mg/day respectively.

7.4.1. Glycaemic control

(a) Fasting plasma glucose

There was no difference in the fasting plasma glucose at diagnosis in the patients randomised at week 0 from metformin and gliclazide therapy. In the two groups there was a similar and significant reduction in the fasting plasma glucose during the dietary run-in period ($p < 0.01$), and after 6 months on metformin or gliclazide therapy ($p < 0.001$) (Tables 7.1 and 7.2).

(b) Glycosylated haemoglobin

Glycosylated haemoglobin in both groups fell during the dietary run-in period ($p < 0.01$) and again after 6 months on metformin or gliclazide therapy ($p<0.001$). There was no significant difference in the HbA_1 between the two groups at any of the three study points (Tables 7.1 and 7.2).

7.4.2. Fasting serum cholesterol and triglycerides

There was no significant difference in the serum cholesterol and triglyceride levels between the two groups at the time of diagnosis, at the end of the dietary run-in periods or after 6 months on oral hypoglycaemic therapy. However, after the

period on diet-alone therapy, there was a significant reduction in both serum cholesterol ($p<0.05$) and serum triglyceride ($p<0.05$) with a further fall in cholesterol ($p<0.01$) and triglyceride ($p<0.05$) after 6 months on oral hypoglycaemic therapy (Tables 7.1 and 7.2).

7.4.3. Body mass index (BMI)

The group randomized to gliclazide had slightly lower BMI at the time of diagnosis compared with those randomised to metformin ($p<0.05$). In both groups the BMI fell during the diet-alone period ($p<0.01$), with the gliclazide group having a slight but significant increase in BMI at the end of 6 months on therapy ($p<0.05$) (Tables 7.1 and 7.2).

7.4.4. Platelet variables (Tables 7.3 and 7.4)

(a) Platelet Count

There was no difference in the platelet count between the diabetic and control groups, and there was no change with therapy.

(b) Platelet Density

At diagnosis the two groups combined had increased platelet density compared with the control group (78.8 (3.0) v 72.8 (2.6); $p< 0.001$). There was no significant difference in the platelet densities between the two groups at diagnosis and there was no significant change by week 0. However, in both the metformin and gliclazide-treated groups there was a significant decrease in platelet density at week 24 compared with the level at diagnosis ($p<0.05$).

(c) Platelet nucleotides (ATP/ADP ratio)

At diagnosis the diabetic groups had increased nucleotide ratio compared with the control group (4.2 (0.9) v 1.7 (0.4); $p<0.001$). By the end of the dietary run-in period there was reduction in the intraplatelet nucleotide ratio ($p<0.001$), with a further reduction after 6 months of oral hypoglycaemic therapy ($p<0.001$).

(d) Intraplatelet ßTG

At diagnosis the intraplatelet ßTG was significantly higher in the combined diabetic group than in the control group (118 (47) v 30 (8.3); $p< 0.001$). There was no significant reduction at the end of the dietary run-in period. However, there was a significant reduction after 6 months of oral hypoglycaemic therapy ($p<0.001$).

(e) Plasma ßTG

At diagnosis the plasma ßTG was significantly higher in the combined diabetic group than in the control group (62 (22) v 29 (9); $p<0.001$). This variable was not measured at week 0. At the end of the 6 months on oral hypoglycaemic therapy, there was a significant reduction compared with at diagnosis levels ($p<0.001$).

(f) Platelet TXB_2

There was no significant difference in the platelet TXB_2 production in response to exogenous collagen between the diabetic group at diagnosis and control group, and there was no alteration with therapy.

(h) Platelet Release Reaction

There was no difference in the platelet release reaction between the diabetic and control groups, and there was no change with therapy.

(i) Intraplatelet cAMP levels

There was no difference in the intraplatelet cAMP response to PGE_1 at diagnosis or after the dietary run-in period between the two groups. However, after 6 months

of oral hypoglycaemic therapy there was a significant increase in levels in both the metformin ($p<0.001$) and gliclazide ($p<0.05$) treated groups 5.

7.4.5. Fibrinogen

The plasma fibrinogen levels were increased in the two diabetic groups compared with controls (3.6 (1.2 v 2.8 (0.7 mg/100ml; $p< 0.005$) and were not significantly altered by therapy.

7.4.6. Side effects

Two patients experienced mild hypoglycaemic episodes on gliclazide and their dosage was reduced. Four of the patients on metformin noticed transient feelings of nausea and mild diarrhoea (2 patients) at the start of treatment, but symptoms lasted only a few days. In order to minimise gastro-intestinal intolerance, the dosage of metformin was increased slowly and the tablets were taken after meals.

Table 7.3

Platelet variables and serum fibrinogen levels in the control and metformin-treated groups at diagnosis (week -3/-6), at the end of the dietary run-in period (week 0) and after 6 months on metformin therapy (week 24).

	Control Group	Metformin Group		
		Week -3/-6	Week 0	Week 24
Platelet count ($x10^9/l$)	280 (67)	319 (113)	303 (91)	295 (45)
Platelet density (pg/ml)	72.8 (2.6)	79.0 (2.9)	78.4 (3.4)	70.8 (4.3)
Platelet neucleotides (ATP/ADP) ratio	1.7 (0.4)	4.3 (1.0)	3.5 (0.9)	2.0 (0.4)
Intraplatelet ßTG (pg/ml)	29.5 (8.3)	121 (54)	98 (37)	44 (23)
Plasma ßTG (µg/ml)	29.1 (8.7)	60.2 (22.4)	- -	46.1 (10.8)
Platelet TXB_2 production (pg/ml)	15.1 (7.2)	17.3 (5.9)	21.8 (11.4)	24.6 (16.2)
Platelet 5HT release (%)	19.4 (6.6)	20.0 (5.8)	18.2 (7.6)	23.0 (5.6)
Intraplatelet cAMP (basal/post PGE1 ratio)	1.1 (0.2)	1.3 (0.3)	1.1 (0.2)	1.5 (0.3)
Fibrinogen (mg/ml)	2.8 (0.7)	3.5 (1.4)	3.4 (1.3)	3.7 (0.8)

Figures expressed as mean (SD)
Platelet density expressed as (x-1) x 1000
See text for significance values

Table 7.4

Platelet variables and serum fibrinogen levels in the control and glicazide-treated groups at diagnosis (week -3/-6), at the end of dietary run-in period (week 0) and after 6 months on gliclazide therapy (week 24)

	Control Group		Glicazide Group	
	Week -3/-6		Week 0	Week 24
Platelet count ($\times 10^9$/l)	280 (67)	299 (57)	287 (35)	302 (40)
Platelet density (pg/ml)	72.8 (2.6)	78.7 (3.2)	78.6 (3.9)	71.2 (4.7)
Platelet neucleotides (ATP/ADP ratio)	1.7 (0.4)	4.2 (0.7)	3.4 (0.7)	2.2 (0.5)
Intra-platelet ßTG (pg/ml)	29.5 (8.3)	115 (41)	98 (37)	54 (22)
Plasma ßTG (µg/ml)	29.1 (8.7)	63.2 (22.8)	- -	42.8 (9.1)
Platelet TBX_2 production (pg/ml)	15.1 (7.2)	19.8 (6.2)	23.3 (10.0)	28.6 (13.3)
Platelet 5HT release (%)	19.4 (6.6)	18.7 (7.5)	18.1 (5.5)	23.9 (6.3)
Intraplatelet cAMP (basal/post PGE_2 ratio)	1.1 (0.2)	1.2 (0.2)	1.2 (0.2)	1.4 (0.2)
Fibrinogen (mg/100ml)	2.8 (0.7)	3.7 (1.1)	3.6 (0.8)	3.6 (0.9)

Figures are expressed as mean (SD)
Platelet density expressed as (x - 1) x 1000
See text for significance values

7.5 Discussion

This study confirmed that both metformin (Clarke and Campbell, 1977; Herman, 1979; Campbell et al, 1987) and gliclazide (Shaw et al, 1985; McAlpine et al, 1988) combined with a diet restricting intake of refined carbohydrate, are effective in the treatment of non- insulin-dependent diabetes. Although the dietary run-in period was effective in reducing both fasting plasma glucose and HbA_1 levels, none of the patients had HbA_1 less than 9% at this stage, and therefore there was no control diabetic group involved in the study. All patients could therefore be regarded as "diet failures". It is feasible that, if the dietary run-in period had been longer (ie 3 - 4 months), a small control group could have been studied (UK Prospective Study, 1983). In both treatment groups, the fasting plasma glucose and HbA_1 levels were equally reduced at the end of the 6 month period, and indeed the mean HbA_1 level in both groups was within the normal range (6 - 8%). Also in both groups, the plasma cholesterol and triglyceride levels fell and it is likely that this, at least in part, was due to the modification in diet. Although there were only small changes in BMI, metformin appeared to be more effective in maintaining the reduced BMI.

This study also demonstrated that platelet abnormalities and increased fibrinogen

levels consistent with hypercoagability are present at diagnosis in non-insulin-dependent diabetic patients (De Silva et al, 1979; Hughes et al, 1983; Peacock et al, 1986). The platelets were denser, contained more alpha- and electron-dense granules, and the increased plasma ßTG was in keeping with increased in vivo platelet activation (Betteridge et al, 1981). It is known that in non-diabetic subjects, dense platelets differ from their lighter less dense cohorts in terms of their content of glycolytic enzymes (Karpatkin, 1974), nucleotides (Booyse et al, 1968) and number of granules (White et al, 1972). In addition they adhere more readily to collagen (Castellan and Steiner, 1976) aggregate more rapidly in the presence of ADP (Karpatkin, 1978) and synthesise protein more actively (Pennington et al, 1976). These dense cells are possibly more immature, and the increase in the overall density of platelets at diagnosis in non-insulin-dependent diabetic patients probably reflects the increased rate of platelet turnover that is known to occur in diabetes (Jones et al, 1981; Tindall et al, 1981). With restriction of the intake of refined carbohydrate, and also metformin and gliclazide therapy, these platelet variables returned towards normal but only the platelet density mean returned to within the normal range. Although gliclazide (Ponasi et al, 1979; Violi et al, 1984) and metformin (Ferugilo et al, 1980; Gin et al, 1988) are reported to have specific effects on human platelets which may be

independent of glycaemic control, they induced very similar platelet changes. In addition, it is interesting to note that the most marked changes in platelet parameters occurred over the 3-6 week period that the patients were on diet therapy alone.

Platelets from diabetic patients have been demonstrated to have reduced sensitivity to inhibiting prostaglandin (Betteridge et al, 1982) and the situation is possibly aggravated in vivo by reduced vascular prostacyclin production (Johnson et al, 1979). Prostaglandin sensitivity is mediated through intraplatelet cAMP levels, and the increase in intraplatelet cAMP with improved glycaemic control seen in this study probably indicates slight increase in platelet prostacyclin sensitivity with improvement in glycaemic control (Janka and Demmel, 1981). Although platelets from insulin-dependent diabetic patients also have increased sensitivity to prostacyclin with improvement of glycaemic control, as discussed in the previous chapter, this is probably mediated through more efficient sequestration of internal free calcium rather than changes in cAMP levels.

Although there was no significant change in platelet TXB_2 production, there was a trend towards an increase in production with improved glycaemic control, possibly

occurring as a result of increased availability of arachidonic acid from membrane phospholipids (Jackson et al, 1984). Similarly there was no difference or change in platelet 5-hydroxytryptamine release, and this probably reflects that this is an insensitive technique to measure platelet aggregation. As reported in previous studies (De Silva, 1979; Cederholm-Williams et al, 1981), the plasma fibrinogen level was elevated in the diabetic group, but there was no change with treatment.

This study demonstrates that platelet abnormalities occur in non-insulin-dependent diabetic patients which may over time contribute to the development of vascular complications (Ganda, 1980; Colwell et al, 1983). These changes are influenced by treatment, although it seems likely that improvement of glycaemic control (Larkins et al, 1988), rather than any specific effect of the oral hypoglycaemic agent employed, is the most important factor in returning these parameters towards normality.

SECTION C

CHANGES IN NEUTROPHIL ELASTASE AND FREE RADICAL ACTIVITY

CHAPTER 8

NEUTROPHIL ACTIVATION DETECTED BY NEUTROPHIL ELASTASE ACTIVITY IN INSULIN-DEPENDENT DIABETES MELLITUS

NEUTROPHIL ACTIVATION DETECTED BY NEUTROPHIL ELASTASE ACTIVITY IN INSULIN-DEPENDENT DIABETES MELLITUS

8.1 Introduction

8.2 Subjects

8.3 Methods

8.3.1 Leucocyte count

8.3.2 Human neutrophil elastase assay

8.3.3 Statistical analysis

8.4 Results

8.4.1 Leucocyte count

8.4.2 Neutrophil elastase

8.4.3 Plasma neutrophil elastase and retinopathy

8.5 Discussion

8.1 Introduction

Recently, increased lysosomal content of neutrophil bactericidal proteins and neutral proteases, have been described in diabetic patients (Oberg et al, 1986). As discussed in earlier chapters, elastase, which is a neutrophil protease, can cause endothelial damage directly (Harlan et al, 1985; Smedley et al, 1986), and it has been suggested that it could contribute to the pathogenesis of vascular damage (Robert et al, 1984). Futhermore, release of neutrophil elastase into the plasma can be used to monitor neutrophil activation and degranulation (Weissman et al, 1980).

The aim of this study was to determine whether plasma (PNE) and/or total (TNE) neutrophil elastase are increased in diabetic patients, and, if so, whether they are associated with microvascular complications in the form of retinopathy.

8.2 Subjects

One hundred insulin-dependent diabetic patients (83 males, 17 females), aged

29.8 ± 4.5 (mean ± SD; range 22-39) years, mean duration of diabetes 12.7 ± 6.6 (1-29) years and 35 comparable non-diabetic control subjects (25 males, 10 females; age 29.6 ± 4.0; range 22-38 years). No patient was known to have active infection. Retinopathy was assessed after mydriasis and was graded as 0 = nil, 1 = microaneurysms or dot haemorrhages only, 2 = exudative charge, 3 = neovascularisation. All subjects were normotensive (BP < 140/90), had normal renal function (plasma creatinine <120 μmol/l, urine Albustix negative) had easily palpable peripheral pulses, and were within 15% of ideal body weight (Metropolitan Life Insurance Tables). Plasma glucose at the time of the study was measured using a Yellow Springs Glucose Oxidase Analyser (Yellow Springs Instrument Co Inc, Yellow Springs, Ohio, USA). Glycaemic control, as assessed by total glycosylated haemoglobin, was measured by electrophoresis using commercially available agar plates (Read et al, 1980), the normal range being 6-8% (Table 8.1).

Table 8.1

Clinical data of diabetic patients and control subjects

Groups	Age (yrs)	Sex	Duration of diabetes (yrs)	Random plasma glucose (nmol/l)	HbA1 (%)	Retinopathy (n) 0	1	2	3
Diabetic Patients	29.8±4.5 (22-39)	83M 17F	12.7±6.6 (1-29)	11.8±5.7 (3.6-27.1)	10.6±2.3 (6.5-17.0)	50	17	17	16
Control Subjects	29.6±4.0 (22-38)	25M 10F	-	-	-	-	-	-	-

Data expressed as mean ± SD with the range in parenthesis

8.3 Methods

8.3.1 Leucocyte count

Peripheral venous blood (15ml) was collected in the morning in the non-fasting state. A full blood count was performed using a Coulter Plus$_\beta$ (Coulter Electronics Ltd, Luton, UK) and a differential leucocyte count was carried out manually on blood films stained with May Grunwald/Geimsa.

8.3.2 Human neutrophil elastase assay

Venous blood for plasma neutrophil elastase (PNE) was anticoagulated with 31.2 g/l trisodium citrate in 50 g/l. Hepes buffer, while 10 ml/l Triton X-100 was added for measurement of total neutrophil elastase (TNE). The sample for PNE was centrifuged at 1500 g for 10 minutes at 4^{o}C and the plasma was then aspirated. Both plasma and lysed whole blood were stored at -20^{o}C and assayed within a week of sampling.

Human neutrophil elastase was measured by a specific radioimmunoassay using

rabbit polyclonal antiserum. The antigen was purified from human neutrophils following leucapharaesis. The antibody was absolutely specific for neutrophil elastase and did not cross react with pancreatic, monocyte or platelet elastase, but measured neutrophil elastase equally well as the free enzyme or as a complex with its inhibitors, alpha-1-proteinase inhibitor and alpha-2-macroglobulin (Greer er al, 1989). In brief, 50 µl of standard/sample was added to 50 µl ^{125}I-elastase (10 $\mu g/l^{-1}$) and 50 µl anti-elastase antibody (dilution 1:3000) and made up to 200 µl with buffer comprising 0.05 mol/l phosphate (pH 7.4), 0.6 mol/l Na Cl, 2 mmol/l EDTA, 130 g/l heparin, 20 kU/l aprotinin and 2% heat inactivated horse serum. Samples were incubated overnight at room temperature and separated with donkey anti-rabbit immunoglobulin immobilised on Sepharose. After shaking for 45 minutes at room temperature the bound complex was separated from the free complex by sedimentation under gravity through a 10% sucrose solution, then aspirated and counted on an NE 1600 gamma counter (Nuclear Enterprises Ltd, Edinburgh, UK). Plasma neutrophil elastase and total neutrophil elastase are expressed as ng/ml and, where relevant, as $\mu g/10^6$ neutrophils. The inter- and intra-assay coefficients of variation were both less than 5%.

8.3.3 Statistical Analysis

Results are expressed as mean ± SD or mean (range) where appropriate. The full blood and polymorph count from the diabetic patients and control subjects were compared using two tailed unpaired Students' t-tests. Plasma neutrophil and total neutrophil elastase from the diabetic retinopathy groups and control subjects were examined by analysis of variance (Student-Newman-Kells Multiple Range Test) after log transformation.

8.4 Results

8.4.1 Leucocyte Count

The total white cell count was slightly, but not significantly increased in the diabetic group when compared to controls. The neutrophil count was significantly greater in the diabetic patients compared with the controls ($P<0.02$) (Table 8.2). However there was no difference in the neutrophil count between the diabetic patients with early and no retinopathy (grades 0 and 1), compared with those with exudative and proliferative retinopathy (grades 2 and 3).

8.4.2 Neutrophil Elastase

Total neutrophil elastase was significantly greater in the diabetic group than in the controls ($p<0.02$). Plasma neutrophil elastase was similarly elevated in the diabetic subjects when compared with controls ($p<0.001$) (Fig 8.1 and Table 8.2).

Total neutrophil elastase ($r = 0.68$; $p < 0.001$), , but not plasma neutrophil elastase showed a significant correlation with peripheral blood neutrophil count (Figs 8.2 and 8.3). When corrected for the neutrophil count, total neutrophil elastase did not differ between diabetic patients and control subjects.

There was no correlation between total neutrophil elastase or neutrophil elastase and age, duration of diabetes, plasma glucose or HbA_1.

8.4.3 Plasma Neutrophil Elastase and Retinopathy

Mean plasma neutrophil elastase was higher in diabetic patients with exudative and proliferative retinopathy (34.98 ng/ml) than in those with little or no retinopathy (25.54 ng/ml). After log transformation of the data, there was no

Table 8.2

White cell count and neutrophil count in the diabetic patients and control subjects.

Groups	White cell count($x10^9$/l)	Neutrophil count($x10^9$/l)
Diabetic patients	7.31(2.07)	5.03(1.60)*
Control subjects	6.59(1.90)	4.33(1.48)

Values expressed as mean (SD).

* $P < 0.05$

Table 8.3

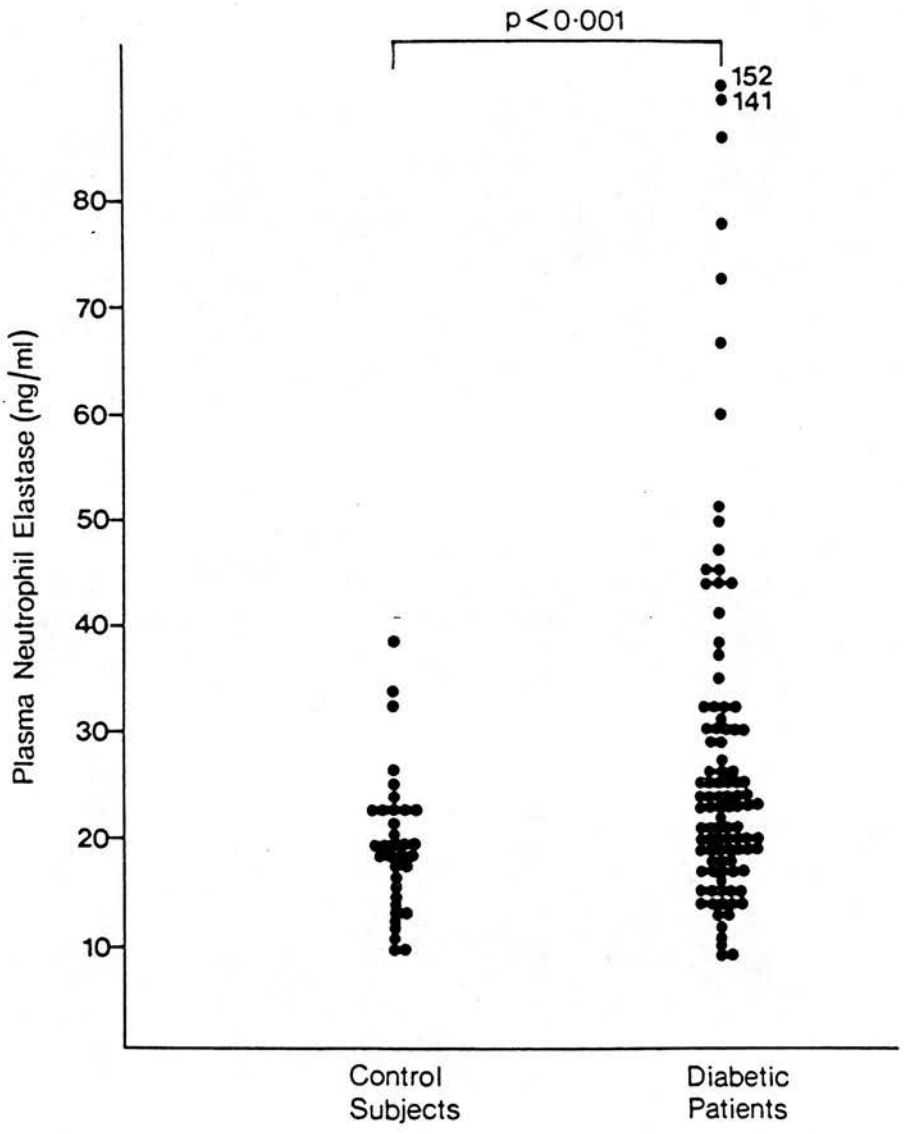
Plasma neutrophil and total neutrophil elastase in the diabetic patients and control subjects

Groups	Plasma Neutrophil Elastase(ng/ml) (PNE)	Total Neutrophil Elastase(ng/ml) (TNE)	TNE/ Polymorph count ($\mu g/10^6$)
Diabetic patients	22.9** (9.4-152.0)	7950* (3070-24000)	1670 (522-3440)
Control subjects	18.5 (9.2-37.8)	6000 (2850-13700)	1450 (950-2540)

Values expressed as median (range)

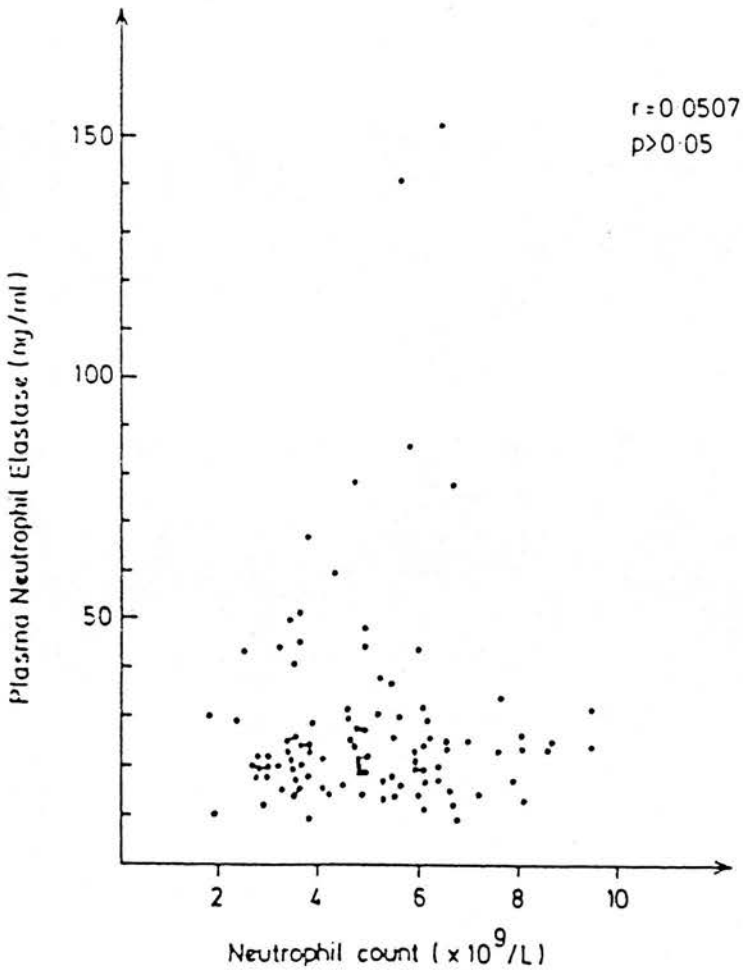
*$p < 0.02$, **$p < 0.001$

Figure 8.1



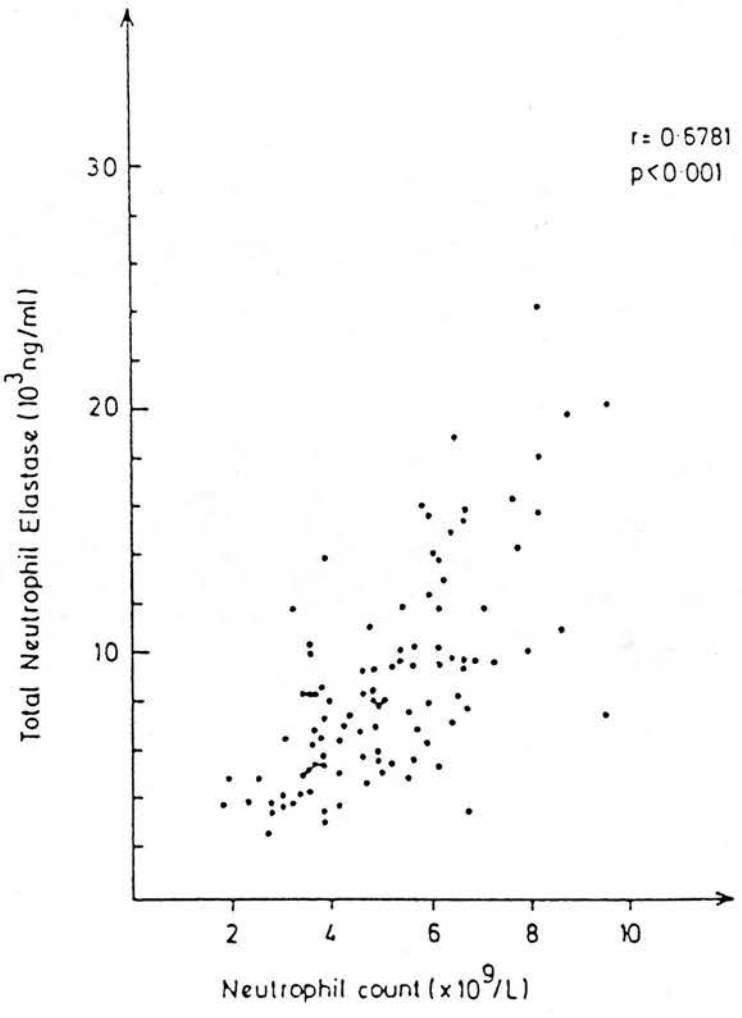
Plasma neutrophil elastase levels in control and diabetic groups

Figure 8.2



Correlation between plasma neutrophil elastase and peripheral neutrophil count

Figure 8.3



Correlation between total neutrophil elastase and peripheral neutrophil count

significant difference between the two diabetic groups. However, of the 20 patients with plasma neutrophil elastase greater than the mean ± 2SD of the control groups, 14 had exudative or proliferative retinopathy.

8.5 Discussion

As discussed in chapter 3, numerous abnormalities of leucocyte function have been described in diabetes. These abnormalities include a reduction in neutrophil killing (Wilson et al, 1986) phagocytosis, bactericidal capacity (Bagdade et al, 1974), and chemotaxis (Mowat and Baum, 1971; Fikrig et al, 1977). These changes, together with local factors such as glycosuria and autonomic bladder dysfunction (Sawers et al, 1986), contribute towards the increased susceptibility to infection seen in diabetes (Bagdade, 1976; Gordon, 1983). Possibly as a reflection of reduced neutrophil efficacy, this study demonstrates that young insulin-dependent diabetic patients, in the absence of overt infection, have a slightly higher neutrophil count than control subjects.

As discussed in earlier chapters, neutrophil elastase is a potent proteolytic enzyme

which is present in high concentrations predominately in the azurophilic granules of neutrophils and myeloid precursors (Janoff, 1985). The assay used in this study is specific for human neutrophil elastase. Polyclonal antiserum raised in rabbits against purified human neutrophil elastase does not bind to any other neutrophil protein, and does not detect antigenically distinct elastases from platelets, monocytes and pancreas. It does however, measure neutrophil elastase equally well in the free form and when complexed to its inhibitors alpha-1-proteinase inhibitor and alpha-2-macroglobulin, the forms in which it circulates in plasma. As neutrophil elastase is largely concentrated in the azurophilic granules of granulocytes, measurement of plasma elastase concentrations provides a marker of neutrophil azurophilic granule release in vivo (Janoff, 1985).

In addition total neutrophil elastase content was increased and correlated strongly with the number of circulating neutrophils. This result, therefore, does not support the previous suggestion that the lysosomal content of elastase is increased (Oberg et al,1986). In contrast there was no correlation between peripheral neutrophil count and plasma neutrophil elastase. This is consistent with the elevation of plasma neutrophil elastase reflecting increased neutrophil activation (Janoff, 1985).

Neutrophils are a vital component of the normal inflammatory response and they are now increasingly implicated in the pathogenesis of certain diseases (Malech and Gallin, 1987; Epstein, 1989). Neutrophil granules contain a range of acid and neutral proteases as well as cationic proteins which increase vascular permeability (Campbell et al, 1982). Neutrophil elastase has a wide range of substrates apart from elastin, for example collagen, immunoglobulin, fibrinogen, complement and proteoglycans (Janoff, 1985). Its ability to degrade collagen and vascular matrix proteins gives it a powerful potential to contribute to the development of vascular damage. The vascular endothelium provides a surface onto which neutrophils can adhere and release their granules directly (Harlan et al, 1985; Smedley et al, 1986). Neutrophil adherence is promoted by endothelial damage and thus in diabetes neutrophil activation and release of neutrophil elastase could occur as a primary phenomenon or alternatively, secondary to endothelial damage due to diabetic angiopathy. The finding in this study that diabetic patients have higher levels of plasma neutrophil elastase, supports the concept of neutrophil-endothelial interaction in the development of diabetic microangiopathy (Oberg et al, 1986). However it does not answer whether release of neutrophil elastase is a primary or secondary phenomenon.

These results also suggest that in addition to the widespread haematological abnormalities in platelets (Colwell et al, 1983), erythrocytes (et al, 1982) and in leucocyte function (Bagdade, 1976; Glass et al, 1987) already recognised in patients with diabetes mellitus, there is also increased neutrophil activation and release of neutrophil elastase which could contribute to the pathogenesis of microvascular disease.

CHAPTER 9

LEUCOCYTE MOBILISATION AND RELEASE OF NEUTROPHIL ELASTASE FOLLOWING ACUTE INSULIN-INDUCED HYPOGLYCAEMIA

LEUCOCYTE MOBILISATION AND RELEASE OF NEUTROPHIL ELASTASE FOLLOWING ACUTE INSULIN-INDUCED HYPOGLYCAEMIA IN NORMAL MAN

9.1 Introduction

9.2 Subjects

9.3 Methods

9.3.1 Serial Sampling

9.3.2 Statistical analysis

9.4 Results

9.4.1 Glucose

9.4.2 Leucocyte and elastase

9.5 Discussion

9.1 Introduction

The brisk counter-regulatory hormonal response to acute insulin-induced hypoglycaemia in normal humans includes the secretion of catecholamines and cortisol (Garber et al, 1976) and these hormones induce a biphasic leucocyte response with an initial rise in the total lymphocyte count followed by a later rise in the granulocyte count (Frier et al, 1986; Fisher et al 1987). The rapid mobilisation of lymphocytes is adrenergically-mediated (Frier et al, 1983; Fisher et al, 1987), while the rise in neutrophils appears to be mediated primarily by cortisol (Corrall et al, 1980; Frier et al, 1986; Fisher et al, 1989). The secretion of catecholamines also causes haemoconcentration with a reduction in plasma volume (Hilsted et al, 1985) which is associated with an acute rise in blood haematocrit (Fisher et al, 1987), an increase in blood viscosity (Neil et al, 1987), platelet activation (Kishikaw et al, 1987) and fibrinolysis (Hutton et al, 1979; Dalsgaard-Nielson et al,1982; Trovati et al, 1986). It has been suggested that when these changes are superimposed on established diabetic microangiopathy, they may encourage intravascular coagulation, reduce capillary blood flow, and precipitate capillary closure, thus aggravating the microvascular complications of diabetes.

As discussed in chapter 8, human neutrophil elastase has been implicated in vascular damage, and total (TNE) and plasma (PNE) neutrophil elastase are elevated in diabetic patients. The aim of this study was to determine whether neutrophil activation manifested by a rise in plasma neutrophil elastase, accompanies the leucocyte mobilisation which occurs in response to insulin-induced hypoglycaemia in non-diabetic subjects, as this may have implications for the contributory role of hypoglycaemic stress to the pathogenesis of diabetic microvascular disease.

9.2 Subjects

Fifteen healthy male normal volunteers, aged 23-39 years were studied in a supine position after an overnight fast. Smoking and the ingestion of caffeine was prohibited for the preceding 12 hours. An intravenous cannula was inserted into an antecubital vein, and after resting for 30 minutes basal blood samples were taken. At time zero, hypoglycaemia was induced using soluble, human insulin (Human Actrapid, Novo Laboratories, Basingstoke, UK) in the dosage of 0.125

units/kg body weight. Plasma glucose was monitored at the bedside at frequent intervals using a glucose meter (Reflolux, BCL, Lewes, UK), and frequent measurements of heart rate and blood pressure were made throughout the study.

9.3 Methods

9.3.1 Serial sampling

Serial blood samples for the measurement of plasma glucose were taken at frequent intervals until 120 min after the acute autonomic reaction (R), which coincided with the nadir of plasma glucose. Sampling was timed from R to account for individual variability in the time of onset of acute hypoglycaemia after the administration of insulin. Blood samples were taken for PNE and TNE at baseline, R + 45 min and at R + 120 min.

Differential blood counts, and PNE and TNE estimations were undertaken as described in chapter 8 .

9.3.2 Statistical Analysis

Results are expressed as mean (SD) or as median (range) where the data is not normally distributed. The data from the three time points were compared using the Wilcoxon rank sum test with the Bonferroni correction.

9.4 Results

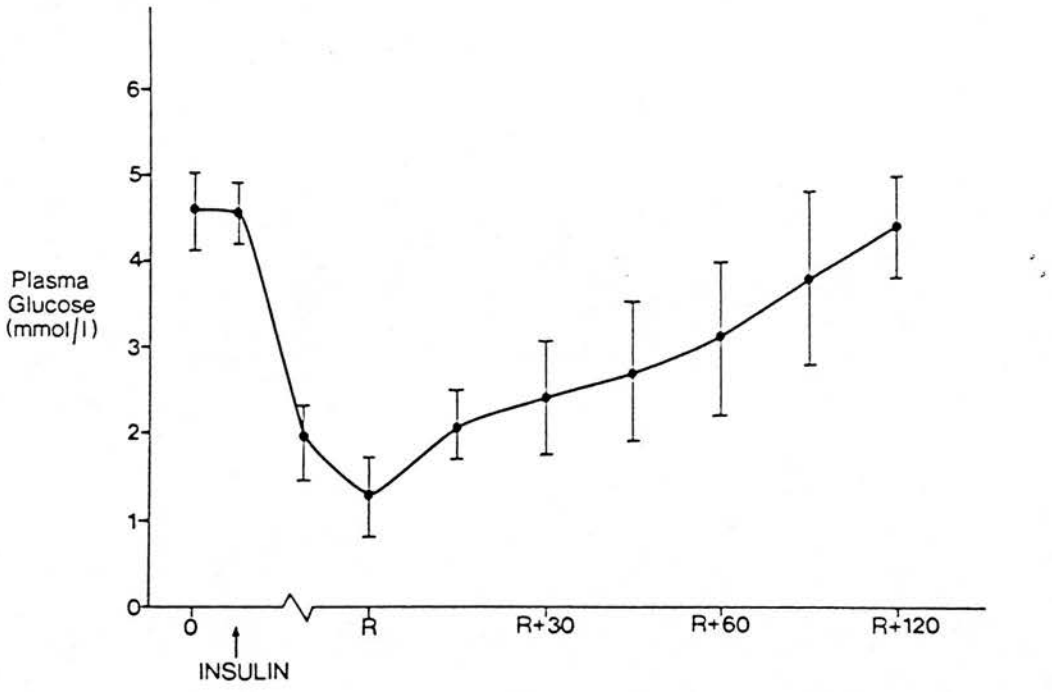
9.4.1 Blood glucose, pulse and blood pressure changes

All subjects experienced an autonomic reaction (R) typical of acute hypoglycaemia with sudden onset of sweating and a tachycardia at approximately approximately 20 (range 18-28) min after the administration of insulin. The plasma glucose fell from a mean (SD) basal value of 4.6 (0.2) mmol/l to a nadir of 1.3 (0.2) mmol/l ($p< 0.01$) which coincided with the onset of R, the acute autonomic reaction, with a subsequent gradual recovery to basal values (Figure 9.1). The changes in pulse and blood pressure were similar to those described previously in normal humans in response to acute hypoglycaemia (Figures 9. 2 and 9. 3) (Frier and Hilsted, 1985; Frier, 1986).

9.4.2 Leucocyte count and elastase levels

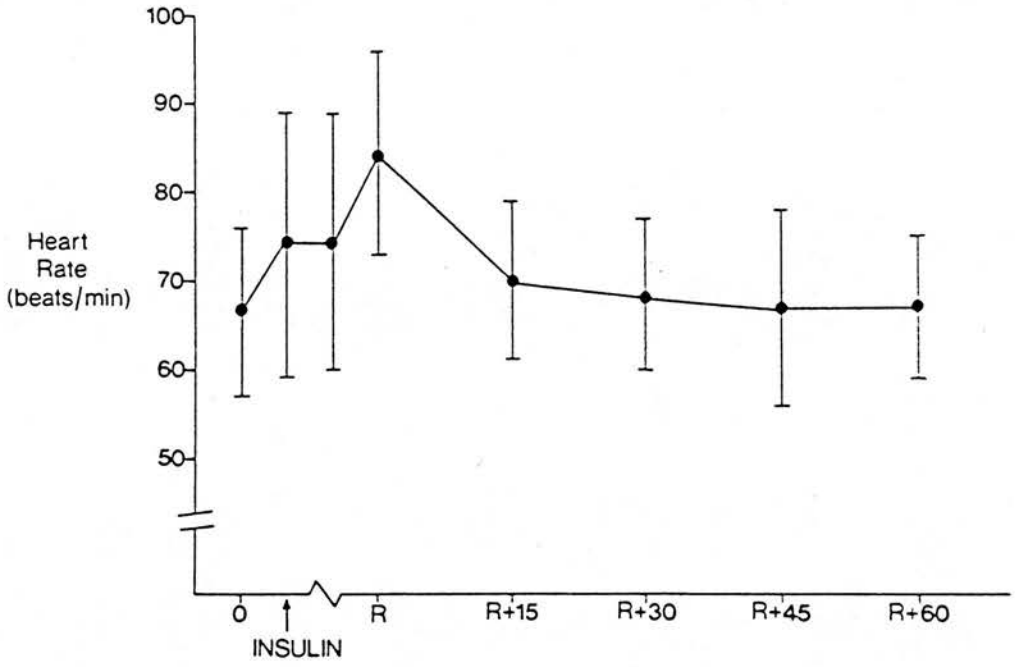
The neutrophil count rose from a median basal value of 3.4 (range 1.9 - 6.5) x 10^9/l to 10.7 (9.4 - 16.3) x 10^9/l at R + 120 min ($p < 0.001$) (Figure 9.4), while the total leucocyte count increased from 5.7 (4.1 - 8.1) x 10^9/l to 12.8 (11.3 - 18.6) x 10^9/l ($p < 0.001$) (Figure 9.5). The plasma neutrophil elastase concentration rose from a basal value of 21 (12 - 34) ng/ml to 29 (14 - 70) ng/ml at R + 120 min ($p< 0.05$) with 11 out of 15 subjects having elevated PNE concentration at R + 120 min. Two subjects had elevation of PNE at R + 45 min, which declined to sub-basal levels at R + 120 min. (Figure 9.6). There was a pronounced rise in total neutrophil elastase from 5900 (3130 - 8200) ng/ml to 25200 (23000 - 52500) ng/ml ($p< 0.001$) (Figure 9.7). A correlation was observed between total neutrophil elastase concentration and neutrophil count before the induction of hypoglycaemia ($r = 0.739$; $p<0.001$), but no correlation was observed between the total neutrophil elastase and the neutrophil count at either R + 45 min or R + 120 min. No correlation was found between the plasma neutrophil elastase and the neutrophil count at any of the three times of measurement.

Figure 9.1



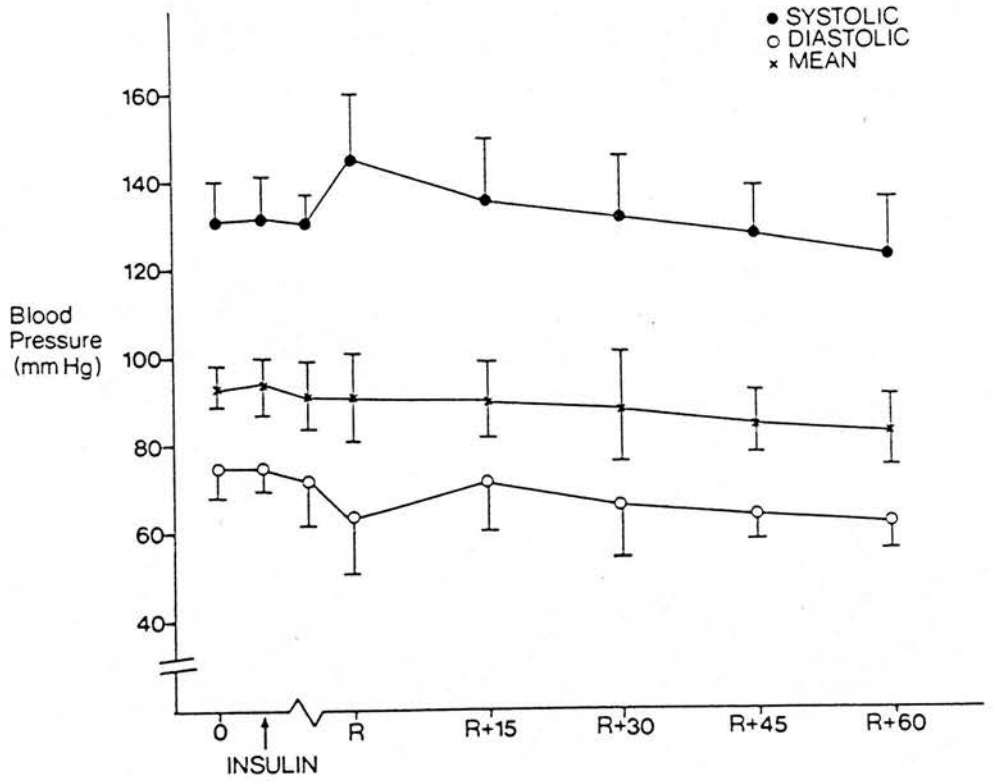
Change in plasma glucose in response to hypoglycaemia. Results expressed as mean ± SD. (Insulin = insulin injection; R = time of hypoglycaemic reaction).

Figure 9.2



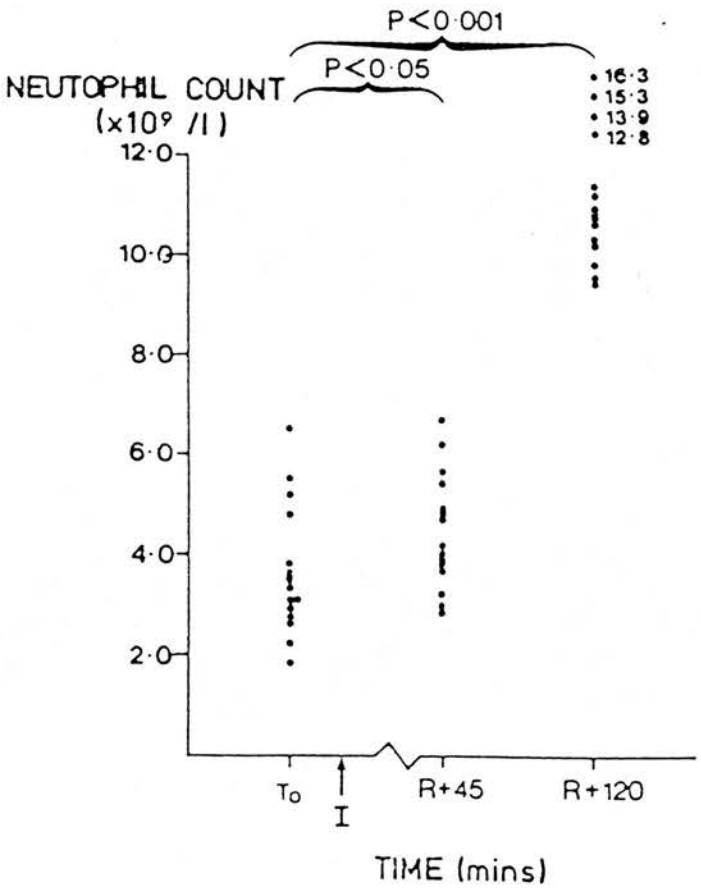
Change in heart rate in response to hypoglycaemia. Results expressed as mean ± SD. (Insulin = insulin injection; R = time of hypoglycaemic reaction).

Figure 9.3



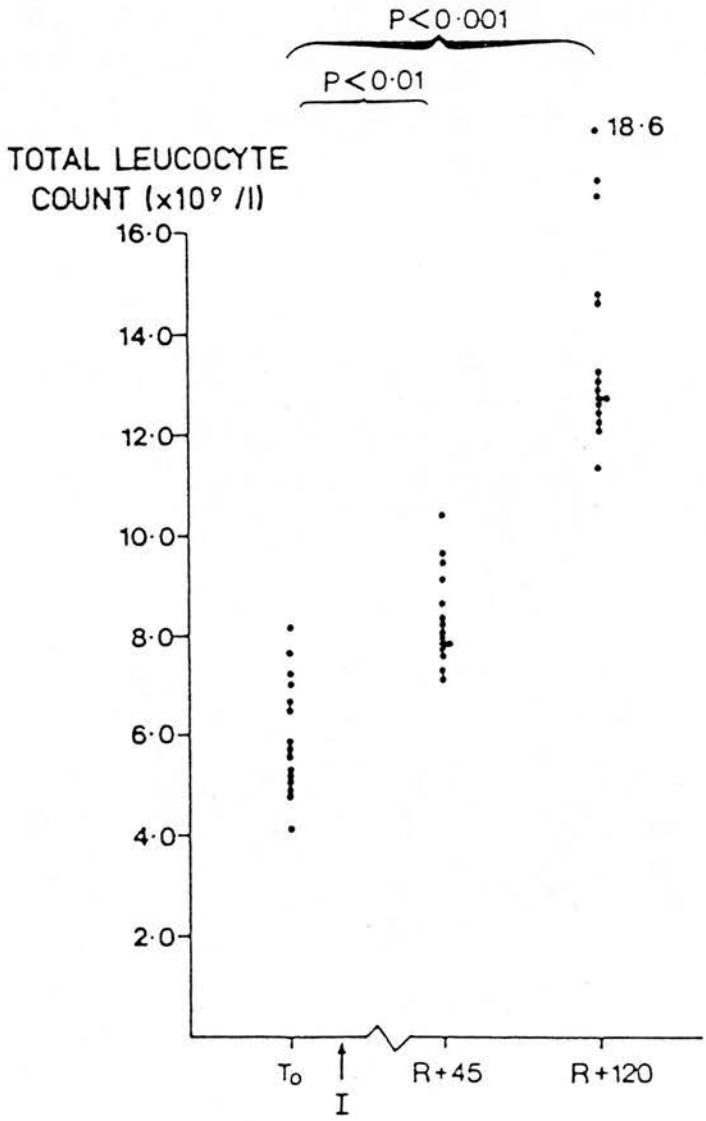
Changes in systolic, diastolic and mean blood pressure in response to hypoglycaemia. Results expressed as mean ± SD. (Insulin = insulin injection; R = time of hypoglycaemic reaction).

Figure 9.4



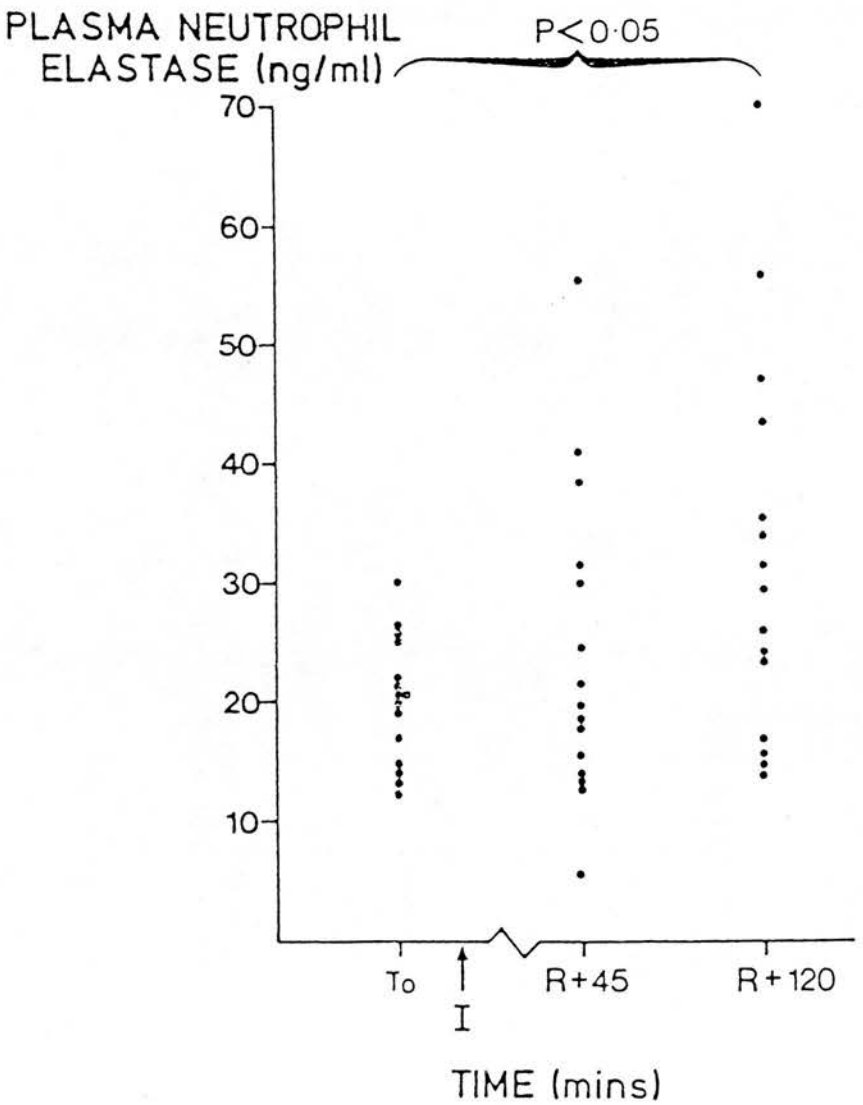
Change in polymorph count in response to hypoglycaemia
(I = insulin injection, R = time of hypoglycaemic reaction)

Figure 9.5



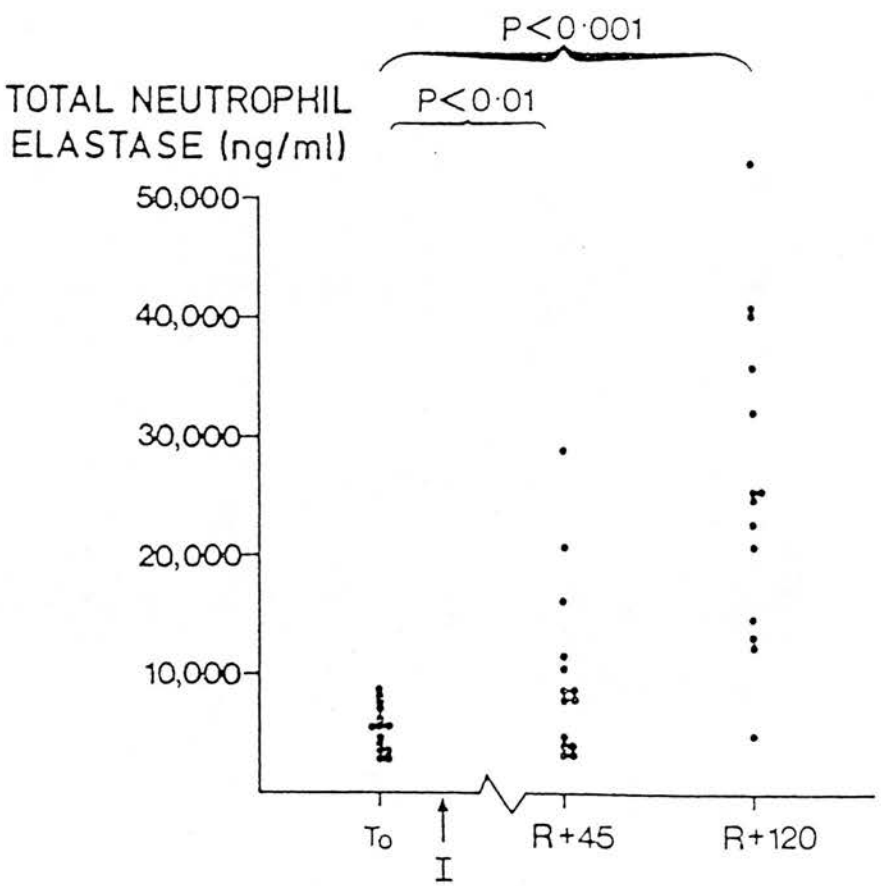
Change in total leucocyte count in response to hypoglycaemia (I = insulin injection, R = time of hypoglycaemic reaction).

Figure 9.6



Change in plasma neutrophil elastase in response to hypoglycaemia. (I = insulin injection, R = time of hypoglycaemic reaction).

Figure 9.7



Change in total neutrophil elastase in response to hypoglycaemia. (I = insulin injection, R = time of hypoglycaemic reaction).

9.5 Discussion

In healthy non-diabetic subjects insulin-induced hypoglycaemia is accompanied by an abrupt increase in heart rate, a rise in systolic blood pressure, and a fall in diastolic pressure (French and Kilpatrick, 1955; Lloyd-Mostyn and Oram, 1975), and cardiac output is increased by about 50% at the onset of hypoglycaemia (Hilsted et al, 1984). Since the mean blood pressure falls only slightly, the calculated total peripheral vascular resistance declines sharply during hypoglycaemia. Vascular resistance is reduced mainly in muscle (Hilsted et al, 1984; Middleton and French, 1974), but in other vascular areas such as the hepatosplanchnic vascular bed it may be unchanged (Hilsted et al, 1984) or decrease only slightly (Beattie et al, 1952). In the subcutaneous vascular bed, vasoconstriction occurs in response to hypoglycaemia both in normal and in diabetic subjects and persists for at least two hours after acute hypoglycaemia (Hilsted et al, 1982). The cardiovascular changes result from stimulation of the sympatho-adrenal system in response to hypoglycaemia and can be largely ascribed to the release of adrenaline from the adrenal medulla, although the precise role of sympathetic innervation remains undefined. It seems likely that vasoconstriction prevails in some areas, although the integrated effect of hypoglycaemia on the

circulation is reduction in vascular resistance (Frier and Hilsted, 1985).

Several parameters of haemostasis alter rapidly in response to acute insulin-induced hypoglycaemia in both diabetic and non-diabetic subjects. These include a rapid rise in factor VIII activity (Corrall et al, 1980), elevation of fibrinogen concentration (Dalsgaard-Neilson et al, 1982), and enhancement of platelet aggregation (Hutton et al, 1979; Hilsted et al, 1980). Plasma ß-thromoglobulin also increases in response to acute hypoglycaemia (Monnier et al, 1985) reflecting increased platelet activation (Kaplan and Owen, 1981). The enhancement of ADP-induced platelet aggregation provoked by hypoglycaemia is associated with a reduction in the platelet count (Hilsted et al, 1980), which further suggests intravascular aggregation. Platelet aggregation during hypoglycaemia appears to be induced by the secretion of adrenaline, which appears to promote aggregation through platelet alpha$_2$-adrenoreceptors (Trovati et al, 1986; Kishikawa et al, 1987). In addition, acute hypoglycaemia provokes an increase in the haematocrit, the erythrocyte count, and haemoglobin concentrations (Frier et al, 1983; Hilsted et al, 1984). As the haematocrit is the principal determinant of blood viscosity, this must also increase acutely in response to hypoglycaemia, thereby influencing capillary blood flow in various vascular beds.

Both hypercoagulability and increased blood viscosity have been implicated in the development of microvascular disease in diabetic patients (Barnes et al, 1977; Lowe et al, 1980).

In this study, a small but significant rise in plasma neutrophil elastase was observed following hypoglycaemia demonstrating that activation of neutrophils occurs in addition to their mobilisation (Frier et al, 1983; Fisher et al, 1987). In addition, hypoglycaemia induced a marked rise in the total neutrophil elastase with loss of the correlation which is usually observed between the neutrophil elastase concentration and neutrophil count. The five-fold increment in total neutrophil elastase was associated with only a three-fold increase in the polymorph count, implying that the mobilised polymorphs have greater overall content of elastase. As elastase is synthesised early in neutrophil development, this observation suggests rapid mobilisation of a younger and less mature population of cells (Gallin, 1984) in response to hypoglycaemia. Hypoglycaemia therefore, through both an increase in neutrophil count and an elevation in elastase concentrations, provides the potential for enhanced leucocyte-endothelial interaction. Furthermore, with the plasma and neutrophil elastase concentration of elastase remaining elevated for 3 hours following acute hypoglycaemia, the

potential for vascular damage persists long after the metabolic recovery from hypoglycaemia. It is possible that insulin-treated diabetic patients who have vascular damage and who are frequently exposed to hypoglycaemia, may have increased vulnerability to the potentially adverse effects of this stress. In these patients neutrophil activation and release of neutrophil elastase may be increased, thus exacerbating pre-existing microvascular disease.

CHAPTER 10

FREE RADICAL ACTIVITY DETECTED BY DIENE CONJUGATES IN INSULIN-DEPENDENT DIABETIC PATIENTS

FREE RADICAL ACTIVITY DETECTED BY DIENE CONJUGATES IN INSULIN-DEPENDENT DIABETIC PATIENTS

10.1 Introduction

10.2 Subjects

10.3 Methods

10.3.1 Glycaemic control

10.3.2 Leucocyte count

10.3.3 Assay of diene conjugates

10.3.4 Statistical analysis

10.4 Results

10.5 Discussion

10.1 Introduction

Indirect experimental evidence has suggested that a free radical mechanism is implicated in the development of vascular disease in diabetes (Jennings et al, 1987). However, the methods for measuring free radical activity have until recently been unsatisfactory (Dormandy and Wickens 1984). Polyunsaturated lipids are particularly sensitive to free radical attack inducing a shift of double bonds to the diene-conjugated configuration (Di Luzzio 1968; Pryor, 1976). Recently the main diene- conjugated compound in human plasma has been identified as the non-peroxide isomer of linoleic acid, PL-9, 11-LA' (Cawood et al, 1983; Iversen et al, 1984; Iversen et al, 1985). In addition, the concentration of this isomer has been shown to vary independently of the main serum lipid classes (Harrison et al, 1985).

The aim of this study therefore, was to measure both linoleic acid (PL-9, 12-LA) and its diene-conjugated non-peroxide isomer (PL-9, 11-LA') by High Performance Liquid Chromatography (HPLC) in a group of insulin-dependent diabetic patients, and to investigate whether any abnormality demonstrated correlated with microvascular disease in the form of retinopathy.

10.2 Patients

Thirty-four insulin-dependent diabetic patients (22 males, 12 females) aged 30.6 ± 4.6 years (mean ± SD), mean duration of diabetes 17.4 ± 4.9 years and 35 comparable non-diabetic controls (25 males, 10 females) aged 29.6 ± 4.9 years were studied. All subjects were normotensive (BP<140/90), and had normal renal function (plasma creatinine <120 μmol/l, urine Albustix negative). Retinopathy was assessed after mydriasis and was graded as 0 = nil, 1 = microaneurysms or dot haemorrhages only, 2 = exudative change, 3 = neovascularisation (Table 1).

10.3 Methods

10.3.1 Glycaemic control

Plasma glucose at the time of the study was measured using a Yellow Springs Glucose Oxidase Analyser. Glycosylated haemoglobin, was measured by electrophoresis using commercially available agar plates (Read et al, 1981), the

Table 10.1

Clinical data of diabetic patients and control subjects

Groups	Age (yrs)	Sex	Duration of diabetes (yrs)	Plasma glucose (mmol/l)	HBA_1 (%)	Retinopathy groups 0	1	2	3
Diabetic Patients	30.6 (4.6)	22M 12F	17.4 (4.9)	12.2 (5.8)	11.1 (2.3)	0	7	13	14
Control Subjects	29.6 (4.9)	25M 10F	-	-	-	-	-	-	-

Values expressed as mean (SD)

For grading of retinopathy see text

normal range being 6 - 8% (Table 10.1).

10.3.2 Leucocyte Count

Peripheral venous blood was collected in the morning in the non-fasting state. Full blood count was performed using a Coulter Plus$_\beta$ (Coulter Electronics Ltd, Luton, UK) and a differential leucocyte count was carried out manually on blood films stained with May- Grunwald/Geimsa.

10.3.3 Assay of Diene Conjugates

Molar concentrations of phospholipid-esterified linoleic acid (PL-9, 12-LA) and of phospholipid-esterified diene-conjugated isomer (PL-9, 11-LA') were measured by the technique described by Iversen et al (1985). 0.5ml of plasma was mixed with 0.5ml of a solution comprising 0.1mol/l Tris (pH 8.9) 1mol/l methanol and 5000 μ/l phospholipase A_2. The mixture was incubated at 25^oC for 15 minutes, after which 2ml methanol containing 0.5% acetic acid and 50mg/l beta-eleostearic acid [18:3 (9,11,13)] was added to precipitate the protein, and the internal standard added. The preparation was then centrifuged and 2ml

supernatant was applied to a "Bond Elut" column that immediately before use was washed twice with 2.5ml propan-2-ol/acetonitrile 2:1 and conditioned twice with 2.5ml of a wash solution of methanol/water/acetic acid 67:33:0.04. The column was again washed after the sample had been applied. The eluate was directly injected into the HPLC by filling the sample loop. The conjugated dienes were measured at 234nm and the non-conjugated fatty acid, linoleic acid, was measured at 205nm.

HPLC was performed on Laboratory Data Control (Stone, Staffordshire, UK) equipment which comprised a Constametic III pump and a Gibson spectromonitor III variable wavelength ultraviolet (uv) detector and an autoanalyser (Cotati, California, USA), which had a 50 µl sample loop. The column used was a spherisorb OD52 250x4mm column containing 5 µm capped spherical particles (Hichrom, Reading, Berks), and had a mobile phase of acetonitrate/water/acetic acid 85:15:0.1 with a flow rate of 1.5ml/min.

10.3.4 Statistical analysis

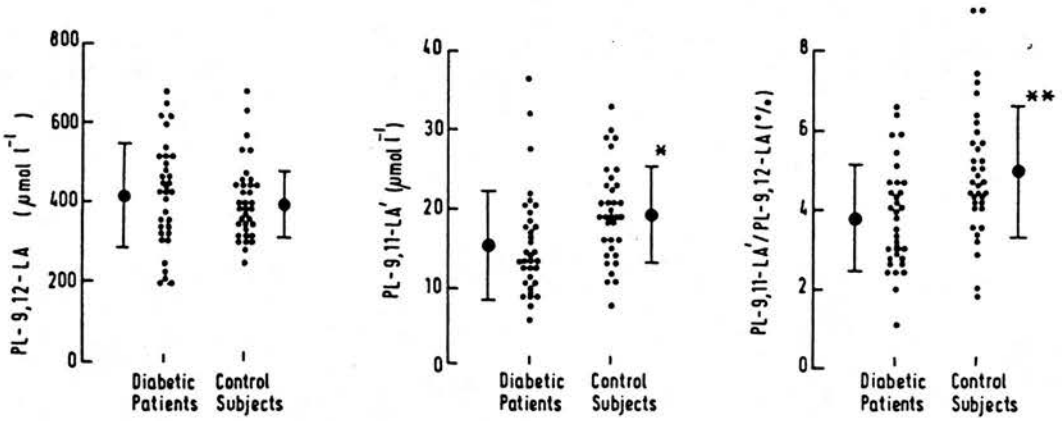
Results are expressed as mean (SD). The data from the diabetic and control groups

were analysed using two tailed unpaired Students' t-tests. Multiple regression analysis was performed to assess the possible influences of the independent variables, HbA_1, and plasma glucose on the concentration of the diene-conjugate of linoleic acid, PL-9,11-LA'.

10.4 Results

There were no differences in the total white count 7.5 (2.1) v 6.9 (1.9) x 10^9/l and the neutrophil count 5.0 (1.5) v 4.3 (1.5) x 10^9/l between the diabetic and control groups. There was also no difference in the molar concentrations of PL-9, 12-LA between the two groups 422 (129) v 402 (81) μmol/l. However, the concentration of PL-9, 11-LA' was significantly reduced in the diabetic group compared with the control group 15.6 (6.7) v 19.3 (3.9) μmol/l ($P< 0.01$); with the molar ratio of PL-9, 11-LA' x 100/PL-9, 12-LA similarly reduced 3.8 (1.3) v 5.0 (1.6) ($P< 0.005$) (Figure 1). The concentration of PL-9, 11-LA', and the ratio of PL-9, 11-LA' to the substrate PL-9, 12-LA in the 14 patients with proliferative retinopathy were 15.6 (7.9) μmol/l and 3.7 (1.3) respectively, and the values were not significantly different from those without neovascularisation. There was no correlation between the HbA1, or plasma glucose

Figure 10.1



The concentration of linoleic acid (PL-9, 12-LA), its non-peroxide diene-conjugated isomer (PL-9, 11-LA') and ratio of PL-9, 11-LA' to PL-9, 12-LA in the diabetic patients and control subjects. Horizontal bars indicate mean $\pm$ SD. (*, $p < 0.01$; **, $p < 0.005$).

and the concentration of PL-9, 11-LA'.

10.5 Discussion

Abnormalities of free radical activity have been implicated in the development of diabetic complications, including vascular disease (Jennings et al, 1987; Croutch et al, 1978; Paller et al, 1984) and cataract development (Srivastava and Ansari, 1988). In addition, the pancreas has been shown to be particularly susceptible to oxidative injury with the islet ß-cell being relatively deficient in enzymes capable of scavenging reactive oxygen radicals (Asayama et al, 1984). Isolated pancreatic cells exposed in vitro to the diabetogenic agent alloxan sustain damage that is preventable by superoxide dismutase, catalase and hydroxyl radical scavengers (Grankvist et al, 1979). There is also evidence that the ability of alloxan to generate reactive oxygen radicals (including hydroxyl radicals) is the primary determinant of its diabetogenic properties (Asayama et al, 1984; Asayama et al, 1984). Futhermore, streptozotocin exerts diabetogenic properties that, like alloxan, may be prevented by prior administration of superoxide dismutase (Gandy et al, 1982), but its actions may not involve the direct

formation of reactive oxygen radicals (Asayama et al, 1984). More recently, it has been demonstrated that tissue antioxidant systems are altered in rats where diabetes is induced by streptozotocin and that these changes are reversed by insulin (Wohaeib and Godin, 1987).

A previous study measuring both oxidation and peroxidation of linoleic acid demonstrated that the summation of these processes is increased in diabetes (Jennings et al, 1987). In non-diabetics, it is recognised that the predominant diene conjugate component of linoleic acid is PL-9, 11-LA' (Iversen et al, 1984). However, this process may be altered in diabetics, and it seems likely that there is a considerable shift to lipid peroxidation (Nishigaki et al, 1981). This reduction in lipid oxidation could be explained by either diminished free radical activity, or by competition between the lipid oxidation and peroxidation pathways. Lipid peroxides themselves have been implicated in vascular endothelial damage (Blake et al, 1985), and they are also intimately involved in prostaglandin biosynthesis; they both stimulate cyclooxygenase and hence prostaglandin synthesis, and inhibit prostacyclin production (Blake et al, 1985; Warso and Lands, 1983; Hemler et al, 1979). This increase in lipid peroxidation may in part explain the abnormalities of platelet and endothelial function (Ham et al, 1979; Colwell et al, 1983) that

have been implicated in the development of diabetic microvascular disease.

As discussed in Chapter 3, the origin of plasma free radical activity is uncertain, although phagocytic cells which produce the free radical anion superoxide particularly during phagocytic killing are a likely source (Nath et al, 1984; Malech and Gallin, 1987). Superoxide is the product of a NADPH-dependent membrane oxidase (Barbior, 1978), which in turn is dependent upon glucose-induced aldose reductase activity. The reduction in bactericidal properties of neutrophils from diabetic patients is due at least in part to the decrease in superoxide production (Wilson et al, 1987), which probably reflects a change in the redox potential within leucocytes secondary to abnormalities of the polyol pathway (Wilson et al, 1987). An alternative explanation for the reduction in free radical activity is increased antioxidant activity, in either serum or membrane structures. However both glutathione and vitamin C, which are potent antioxidants, have been demonstrated to be reduced in diabetic patients (Chari et al, 1984; Jennings et al, 1987).

Although free radicals may have a role in the microenvironment of the vascular endothelium, these results do not support the hypothesis that free radicals play a

Although free radicals may have a role in the microenvironment of the vascular endothelium, these results do not support the hypothesis that free radicals play a significant role in the development of diabetic vascular disease. Indeed, if the reduction in free radical activity is secondary to polyol pathway abnormalities, and yet they do play a part in the development of diabetic vascular disease, treatment with an aldose reductase inhibitor (Stribling and Perkins, 1986), may aggravate vascular disease.

SECTION D

CONCLUSIONS

CHAPTER 11

CONCLUSIONS

Although microvascular disease will not occur in the absence of hyperglycaemia, there is wide individual variation in the time of appearance and severity of microvascular complications. For example, approximately one-third of patients with insulin-dependent diabetes mellitus will develop severe complications, whereas the other two-thirds run a more benign course (Deckert et al, 1978). In addition, about 20% of insulin-dependent diabetic patients do not develop clinically evident complications even after 40 years of diabetes, yet a small minority may have severe problems after only five years without any obvious difference in glycaemic control (Rosenstock and Raskin, 1988). It would seem likely that some patients demonstrate a genetic susceptibility, although the relevant genetic markers and their roles remain unresolved (Jennings and Barnett, 1988).

Previous studies in diabetes have demonstrated numerous abnormalities of platelet and leucocyte function and also free radical activity. However, the results are often inconsistent and conflicting. This has occurred, at least in part, because of the heterogeneity of the groups of patients studied and the diverse methods used. In addition, conclusions are often inferred from indirect methods. Furthermore, studies tend to investigate only one aspect, while it seems likely that there is considerable interaction between platelet, leucocyte, endothelial and hormonal

abnormalities (Stout, 1979; Ganda, 1980; Colwell et al, 1983; Oberg et al, 1986).

The work in this thesis supports the concept of platelet and leucocyte activation contributing to the development of diabetic microvascular disease. In the majority of studies described, the microvascular complication, retinopathy, has been investigated. As far as possible, the diabetic patients have been clinically free of macrovascular disease, as it is probable that the underlying aetiopathogenesis for macrovascular disease is quite different. In addition, the insulin- and non-insulin-dependent diabetic patients studied have been in homogeneous groups and have been investigated using modern methodology. The platelet abnormalities demonstrated include changes in density, increased thromboxane production and sensitivity, and decreased sensitivity to prostacyclin. These abnormalities, along with the other changes in platelet function demonstrated, are consistent with the hypercoagulable state that exists in diabetes. Although there were subtle differences in the changes shown in insulin-dependent diabetes compared with non-insulin-dependent diabetes, it is presumed that the underlying aetiopathogenesis is similar for the two types of diabetes.

The increase in neutrophil elastase levels demonstrated, particularly during insulin-induced hypoglycaemia, is also consistent with leucocyte-endothelial interaction contributing to diabetic microvascular disease. Although the plasma level of free radical activity, as shown by the level of the diene-conjugate of linoleic acid (PL-9, 11-LA'), was not increased in insulin-dependent diabetes, abnormalities of lipid metabolism related to free radical activity may still contribute to diabetic microvascular disease. Furthermore, as discussed in Chapter 10, the possible shift to lipid peroxidation in diabetes may interfere with prostaglandin biosynthesis, resulting in altered platelet and endothelial function. In addition, lipid peroxidation may also interfere with the action of alpha-1-proteinase inhibitor, thereby increasing the potential for neutrophil elastase to cause vascular damage.

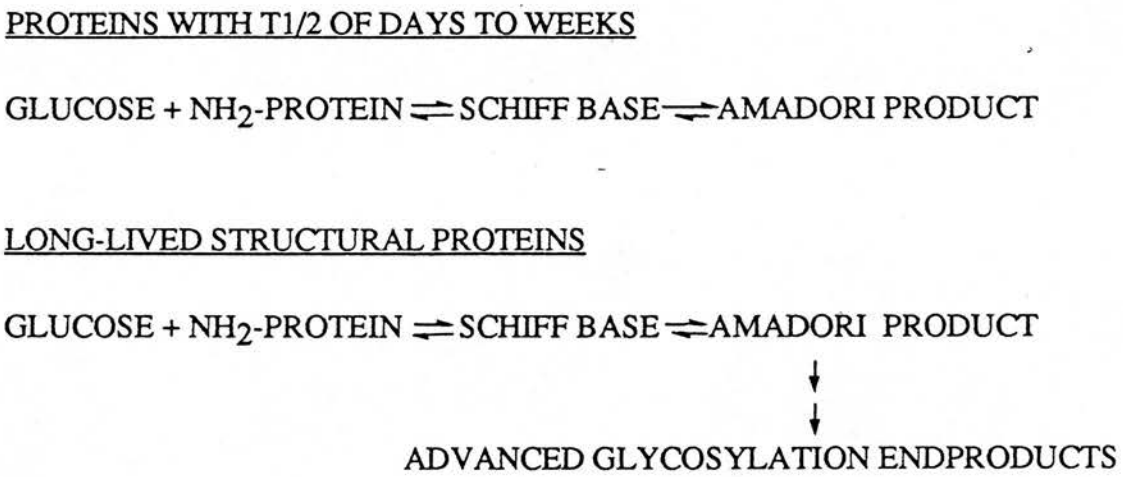
Why abnormalities of platelet, leucocyte and endothelial function should occur in diabetes remains unclear. Furthermore, it remains to be resolved whether the changes occur as a primary phenomenon or, alternatively, secondary to endothelial damage due to diabetic microvascular disease. It would seem likely, however, that although some of the abnormalities result from vascular disease, the majority contribute to and aggravate diabetic vascular disease (Colwell et al, 1983).

There appear to be two possible general pathophysiological mechanisms by which hyperglycaemia leads to irreversible tissue damage. A major consequence of hyperglycaemia is excessive non-enzymatic glycosylation of proteins (NEG) (Eble et al, 1983; Brownlee et al, 1984; Kennedy and Baynes, 1984). The initial step in NEG is rapid and reversible, and results in the formation of a Schiff base. This is followed by a slow rearrangement to a more stable ketoamine linkage (the Amadori rearrangement). With further reactions and rearrangements beyond the Amadori product, advanced glycosylation end-products accumulate (Monnier et al, 1984) causing increased cross-linkage of proteins such as collagen (Figure 11.1). In connective tissue, brown fluorescent pigments appear (Monnier et al, 1986) and collagen exhibits decreased susceptibility to in vivo and in vitro proteolysis (Lubec and Pollak, 1980) with increased stiffness and thermal stability (Andreassen et al, 1981; Sternberg et al, 1985). Although circulating cells have considerably shorter half-life than collagen it seems likely that they are affected by NEG leading to possible alterations in function. In particular, changes in platelet and leucocyte enzyme activity along with binding of regulatory molecules may result from NEG (Brownlee et al, 1984). In addition, relevant to platelet behaviour, collagen extracted from placentae of diabetic patients has increased ketoamine-linked glucose, and such collagen exhibits increased platelet-

aggregating ability (Le Pape et al, 1983).

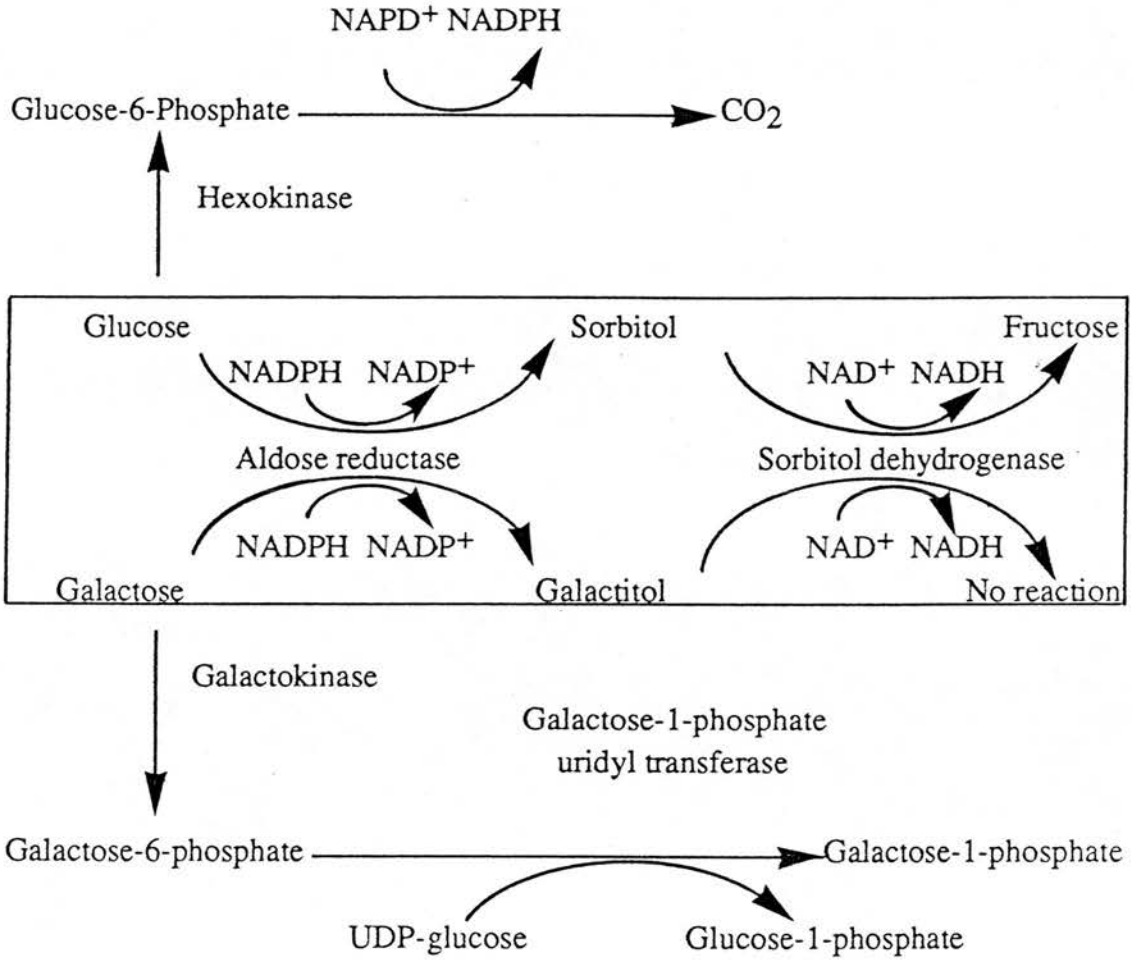
The other major consequence of hyperglycaemia is induction of altered steady-state levels of intracellular metabolites. Aldose reductase and sorbitol dehydrogenase form the two-step sorbitol pathway through which glucose is converted to fructose. In the first step aldose reductase utilizes NADPH to catalyse the stereospecific reduction of glucose to sorbitol (Figure 11.2). In most tissues the cellular accumulation of sorbitol is negligible; however in diabetes, increased intracellular concentrations of sorbitol lead to adverse effects, including increased osmotic activity, especially in the lens, causing increased influx of fluid and changes in membrane permeability, and subsequent onset of cellular pathology (Kinoshita, 1974; Stribling and Perkins, 1986). Increased levels of sorbitol are also associated with a parallel decreased concentration of myo-inositol, and altered cellular redox potentials result from the rapid depletion of NADPH (Gonzalez et al, 1984; Greene et al, 1987). In relation to diabetic vascular disease, abnormalities of the polyol pathway have been particularly implicated in basement membrane thickening (Kador and Kinoshita, 1985) and increased vascular permeability (Lightman et al, 1987). Consistent with this concept, in animal models elevation of the plasma level of the isomer of glucose, D-galactose, can induce changes in the polyol pathway (Figure 11.2). This may reproduce many of the effects of diabetes

Figure 11.1



Formation of early reversible and advanced irreversible non-enzymatic glycosylation endproducts.

Figure 11.2



The polyol pathway in diabetes mellitus

on peripheral nerves, lens, cornea and retina (Datiles et al, 1983; Kinoshita, 1974; Engerman and Kern, 1984).

Diabetic microvascular disease clearly results from highly complex events, which appear to be regulated by an array of environmental and possibly genetic influences (Ganda, 1980). Full understanding of the role of the many factors involved will only be achieved by long-term prospective studies following patients from the onset of disease to the development of complications. These studies would give greater insight if they included intervention limbs. At the present time, the main clinical method for delaying the onset of diabetic vascular disease, is the maintenance of good glycaemic control. Indeed it has been demonstrated that improving metabolic control to produce "near-normoglycaemia" may retard or arrest the progression of diabetic microangiopathy (Hanssen et al, 1986). Unfortunately, good glycaemic control is often associated with loss of "hypoglycaemic awareness" (Lager et al, 1986, Amiel et al, 1987), and continuous subcutaneous insulin infusion can be complicated by hyperkalaemia and ketoacidosis (Hanssen et al, 1986). In addition, particularly as a result of "hypoglycaemic unawareness", very "tight" glycaemic control can not be applicable to all patients.

Non-steroidal anti-inflammatory drugs have been shown to delay the onset of and to decrease the rate of progression of microvascular disease (Powell and Field, 1964; Colwell et al, 1986; Donadio et al, 1988; The Damad Study Group, 1989). The anti-prostaglandin agents studied lacked specificity and they probably effect both TXA_2 and PGI_2 synthesis. It is probable that a specific thromboxane synthetase inhibitor may be more appropriate, and consistent with this a thromboxane synthetase inhibitor has been shown to reduce microalbuminuria in insulin-dependent diabetic patients (Barnett et al, 1984).

In addition, antioxidants protect against free radical damage. The nutritional antioxidants vitamin C and E are reduced in diabetes, and the increase in dietary intake may be of therapeutic advantage and also allow for the further evaluation of the role of free radicals in the pathogenesis of microangiopathy (McCarthy and Rubin, 1984).

Aldose reductase inhibitors have been extensively investigated in both animal models of vascular complications and in human diabetes. As suggested in Chapter 10, aldose reductase inhibitors may increase free radical activity. However, through NADPH-sparing effects, there may be an increase in antioxidant reserve

allowing tissues to be better able to deal with oxidative stress (Barnett et al, 1986).

Recently it has been reported that aminoguanidine, a nucleophilic hydrazine compound, prevented both the formation of fluorescent advanced glycosylation end-products and the formation of glucose-derived collagen cross-linking both in vitro and in vivo (Brownlee et al, 1986). This compound prevents protein browning and also the generation of superoxide radicals (Jones et al, 1986). Therefore, aminoguanidine or a similar compound, may be important in the investigation of the role played by extensive glycosylation-induced protein cross-linking in the development of microangiopathy.

The use of specific therapeutic intervention, that does not rely solely on producing near-normoglycaemia, could therefore be of therapeutic importance. Furthermore, this form of intervention would probably lead to greater understanding of the aetiopathogenesis of diabetic microvascular disease.

LIST OF PUBLICATIONS FROM M D THESIS

Original Articles

1. Collier A, Watson HHK, Strain L, Ludlam CA, Clarke BF. Platelet density analysis and intreplatelet granule content in young insulin-dependent diabetics. Diabetes 1986; 35: 1081-1084

2. Collier A, Tymkewycz PM, Armstrong R, Young RJ, Jones RL, Clarke BF. Platelet thromboxane sensitivity in insulin-dependent diabetic patients with proliferative retinopathy. Diabetologia 1986; 29:471-474

3. Collier A, Tymkewycz PM, Mathews DM, Jones RL, Clarke BF. Increased platelet sensitivity to inhibiting prostaglandins and thromboxane production in insulin- dependent diabetics with short term improvement of glycaemic control. Diabetes Res 1987;5:79-82

4. Collier A, Jackson M, Dawkes RM, Bell D, Clarke BF. Free radical activity detected by diene conjugates in insulin-dependent diabetic patients. Diabetic Med 1988; 5:747-749

5. Collier A, Jackson M, Bell D, Patrick AW, Mathews DM, Young RJ, Clarke BF, Dawes J. Increased neutrophil elastase activity in insulin-dependent diabetes mellitus. Diabetes Res 1989; 9: 117-120.

6. Collier A, Watson HHK, Patrick AW, Ludlam CA, Clarke BF. Effect of glycaemic control, metformin and gliclazide on platelet density and aggregation in recently diagnosed Type 2 (non-insulin-dependent) diabetic patients. Diabété Metab (In Press).

7. Collier A, Patrick AW, Hepburn D, Bell D, Jackson M, Dawes J, Frier BM. Leucocyte mobilisation and release of neutrophil elastase following acute insulin-induced hypoglycaemia in normal humans. Diabetic Med (In Press)

Presentations to Scientific Meetings

1. Collier A, Watson HHK, Strain L, Matthews DM, Clarke BF, Ludlam CA.
   Density analysis of platelets in diabetes mellitus.
   British Diabetic Association, March 1985
   Diabetic Med 1985; 2 : 309

2. Clarke BF, Collier A, Jones RL, Matthews DM, Tymkewycz PM.
   Changes in platelet function in diabetes mellitus with improvement of glycaemic control.
   British Pharmacological Society, September 1985
   Br J Pharm 1985; 86:795

3. Collier A, Tymkowycz PM, Armstrong R, Young RJ, Jones RL, Clarke BF.
   Platelet thromboxane production and receptor sensitivity in diabetic proliferative retinopathy.
   British Diabetic Association, April 1986
   Diabetic Med 1986; 3:358

4. Collier A, Jackson M, Bell D, Patrick AW, Matthews DM, Young RJ, Clarke BF, Dawes J.Increased neutrophil elastase activity in insulin-dependent diabetes mellitus
   European Association for the Study of Diabetes, September 1987
   Diabetologia 1987; 30:50

5. Collier A, Jackson M, Dawkes RA, Bell D, Clarke BF.
   Reduced free radical activity detected by diene conjugates in insulin-dependent diabetic patients.
   British Diabetic Association, September 1988
   Diabetic Med 1988; 5:18

6. Collier A, Watson HHK, Patrick AW, Ludlam CA, Clarke BF.
   Effect of metformin and gliclazide on platelet density and aggregation in recently diagnosed Type 2 (non-insulin-dependent) diabetic patients.
   British Diabetic Association, April 1989
   Diabetic Med 1989; 6: 26

7. Jackson M, Collier A, Dawes J, Muir AL, Bell D.
White cell count and neutrophil elastase are elevated in patients with vascular disease.
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